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APPENDIX C

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CORRELATIONS BETWEEN THE *IN VITRO* AND *IN VIVO* ACTIVITY OF ANTI-HIV AGENTS: IMPLICATIONS FOR FUTURE DRUG DEVELOPMENT

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Some 10 years after the first recognition of acquired immunodeficiency syndrome (AIDS) as a new syndrome, we have identified a number of molecular targets to interrupt the replicative cycle of human immunodeficiency virus (HIV), the causative agent. A number of didcoxynucleosides have been identified as having anti-HIV activity *in vitro*, and several of these have been found to have clinical activity in patients. In contrast, while a number of agents have been found to block viral binding to the target cell *in vitro*, these agents have generally not shown clear-cut evidence of clinical activity. Agents which act at a variety of steps in the HIV replicative cycle are now under development, and it is likely that we will have an increased armamentarium to fight this disease in the near future.

KEY WORDS: Anti-HIV agents, CD4, AZT, zidovudine, ddC, ddI, reverse transcriptase, HIV-protease.

1. INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) epidemic is now in its second decade. Since AIDS was first recognized as a new disease entity in 1981, the causative agent, human immunodeficiency virus (HIV), has been identified and we have accumulated a substantial understanding of the replicative cycle of this agent. HIV is perhaps the most complex retrovirus studied; it thus offers a variety of potential points of attack[1] (Table I). Several antiretroviral drugs have now been developed,[2] and coupled with better therapy for opportunistic infections, the expected survival of AIDS patients today is consequently longer than it was at the beginning of the epidemic.[3,4] In addition, the use of anti-retroviral therapy in earlier stages of HIV infection has been shown to delay the progression to frank AIDS, both in controlled randomized clinical trials and in epidemiologic studies.[5–8] However, AIDS remains a fatal illness. No curative treatment is available, and there is an urgent need for improved therapeutic strategies. As such, it is perhaps worthwhile to consider the experience to date with certain molecular targets for anti-HIV therapy, to help guide us in developing future approaches.

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TABLE I
Steps in the HIV Replicative Cycle which may be Targets for Therapy

Step	Possible intervention
Binding to target cell	CD4 analogues Antibodies to HIV or receptor Non-specific inhibitors (e.g. dextran polysulfate)
Fusion to target cell	Anti-gp41 antibodies Drugs which block fusion
Entry and uncoating of RNA	Drugs to block this step of HIV replication may be found
RNA to DNA transcription by reverse transcriptase	Many active drugs (e.g. AZT and other dideoxynucleosides, phosphonoformate, TIBO derivatives) act at this step
Degradation of RNA by RNase	Specific inhibitors of HIV RNase may be found
Migration to nucleus and integration into host DNA	Agents which inhibit these steps may be found
Transcription and translation	Inhibitors of Tat or Rev activity Anti-sense constructs? (e.g. against *tat* or *rev*) TAR decoys (which may bind Tat) Ribozymes may destroy HIV mRNA
Ribosomal frameshifting	Inhibitors of frameshifting may be found
Cleavage of Gag and Pol polyproteins	A number of inhibitors of HIV protease have been developed
Protein modification	Glycosylation inhibitors (e.g. castanospermine) Myristoylation inhibitors may be found
Viral packaging	Antisense constructs against the packaging sequence may be found to have activity
Viral budding	Interferons (may work at other steps as well) Antibodies to viral release antigens Agents which selectively kill cells expressing HIV antigens (e.g. CD4-toxin fusion proteins)

2. REVERSE TRANSCRIPTASE INHIBITORS

2.1. Dideoxynucleosides

Reverse transcriptase, an enzyme encoded by the *pol* gene of HIV, is essential for HIV replication and is for all practical purposes a unique viral enzyme. Soon after HIV was found to be the causative agent for AIDS, several members of the family of compounds known as dideoxynucleosides were found to be potent and selective anti-HIV agents *in vitro*.[1,9,10] The development of such drugs depended on the establishment of assays employing live AIDS virus in human cells. A number of these compounds have subsequently been shown to have clinical activity in HIV-infected patients.[11-18] This work was able to proceed so rapidly in part because of the earlier pioneering work of Horwitz, Ostertag, Furmanski, and others in the 1960's and 1970's.[19-23] Dideoxynucleosides undergo anabolic phosphorylation in target cells to the active 5′-triphosphate moieties.[2] (The specific metabolic pathways vary from drug to drug.) In a fully phosphorylated form, they inhibit viral polymerase (reverse transcriptase) activity both by acting as chain terminators and as competitive inhibitors of deoxynucleoside-5′-triphosphates.[24,25] The phosphorylation of dideoxynucleosides to their active moieties is catalyzed by mammalian kinases (HIV is not known to encode for such kinases).

As a result, there are substantial species differences in the anabolic catabolism of dideoxynucleosides, and this is one reason why it is difficult to predict their activity against HIV in human cells from experiments in murine systems. For example, the drug ddC is poorly phosphorylated in murine cells and has little activity against murine retroviruses in such cells; in contrast, it is efficiently phosphorylated in human T cells and is one of the most potent anti-HIV agents *in vitro* in such cells.[10,26] In human cells, different sets of enzymes catalyze the phosphorylation of the various dideoxynucleosides.[2] Thus, these drugs have different activity and toxicity profiles, and each has to be considered on its own terms as a separate agent.

At least 7 dideoxynucleosides, including 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine), 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyinosine (ddI, didanosine), have now been administered to patients, and several of these have been found to have clinical anti-HIV activity.[11-18,27,28] At this point, one can say that within this class of drugs, potent and selective *in vivo* activity in certain systems using HIV and human target cells can be a fairly good predictor of clinical activity (unless unexpected toxicity prevents administration at adequate doses). Animal toxicity studies have not been reliable predictors of the principal toxicities of this class of drugs. For example, peripheral neuropathy (the dose-limiting toxicity of ddC) and pancreatitis (an important dose-limiting toxicity of ddI) were not observed in preclinical animal toxicity studies (although animal models of neuropathy have subsequently been developed). This again serves as a reminder that the anabolic phosphorylation of dideoxynucleosides varies from species to species, and one must use caution in extrapolating from lower animals to man for the selection of anti-retroviral agents.

The first three dideoxynucleosides to be extensively analyzed in patients were AZT, ddC, and ddI.[11-18] All were found to induce at least transient increases in the number of CD4 cells and decreases in HIV p24 antigen in patients with AIDS or AIDS-related complex. In the case of two of these drugs, AZT and ddI, the drug levels associated with clinical activity were consistent with those that had anti-HIV activity *in vitro*.[9,10] In contrast, ddC (Figure 1) appeared to be somewhat more potent in patients than may have been predicted from certain pre-clinical studies. Moreover, this compound appeared to have a relatively greater effect on serum HIV p24 antigen than on the CD4 count.[13,14] The factors responsible for this quantitatively and qualitatively different pattern of activity are not understood at present. One possibility is that even closely related drugs may exhibit differential effects in various target reservoirs of virus (T cells, monocytes, etc.). As will be discussed below, preliminary results suggest (but do not prove) that combination regimens of AZT and ddC have more activity (and less toxicity) in patients than either drug used alone, and it is possible that this drug will find its greatest usefulness in combination therapy.[29,30]

The potent *in vivo* activity of ddC in patients has spurred interest in related compounds. Two ddC analogues, 2′-3′-dideoxy-3′-thiacytidine (also called 3′-thiacytidine or 3TC)[31] and 2′-β-fluoro-2′,3′-dideoxycytidine (also called 2′-fluoro-2′,3′-arabinofuranosyl cytosine or 2′-*threo*-FddC),[32,33,34] have both been found to have potent anti-HIV activity *in vitro*, and these compounds are now being studied in Phase I trials (Figure 1). It will be of interest to compare their activity and toxicity profiles to that of ddC. Two other ddC analogues, 2′,3′-didehydro-2′,3′-dideoxycytidine (also called 2′,3′-dideoxycytidinene) and 2′,3′-β-epoxy-2′,3′-dideoxycytidine, are also active against HIV *in vitro* (Figure 1).[35,36] By the same token, certain fluoro- or amino-substituted analogues of dideoxypurines[37,38] and lipophilic halogenated

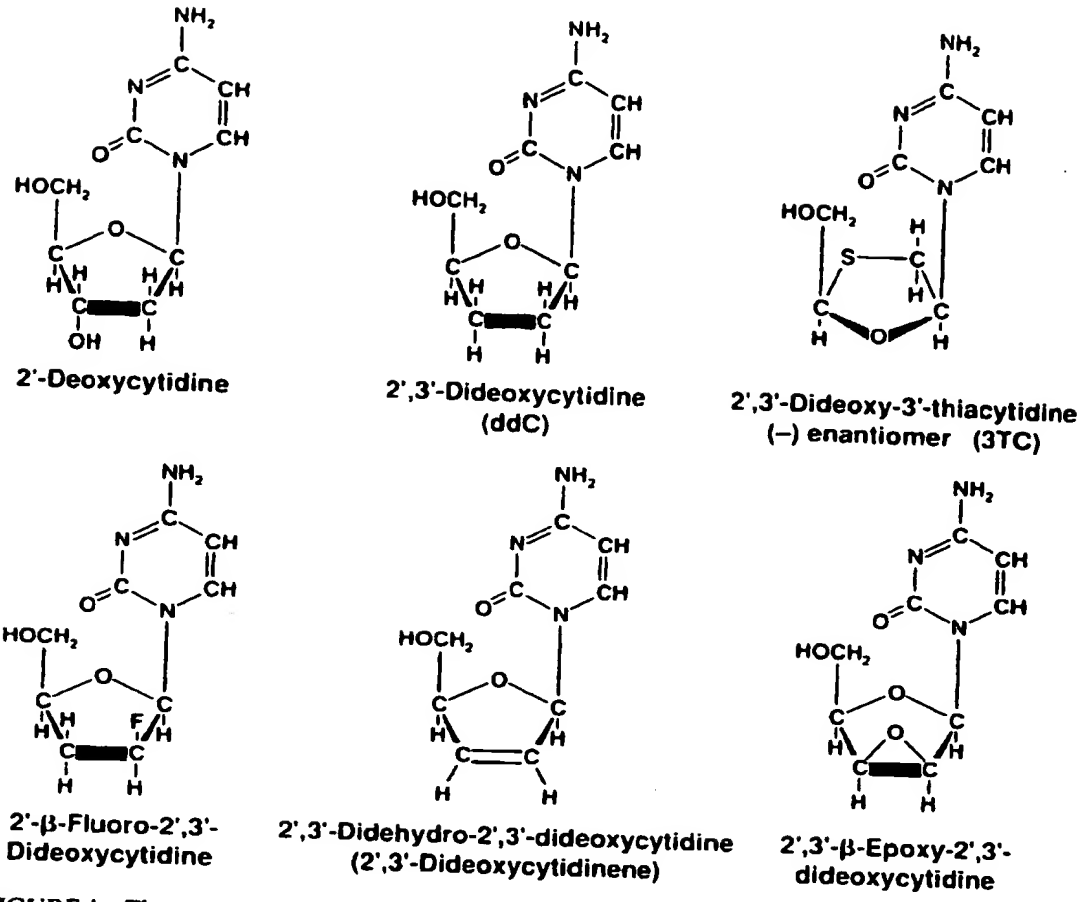

FIGURE 1 The structures of 2'-deoxycytidine (top left) and 5 analogues with potent and selective anti-HIV activity *in vitro*. Three of these analogues, ddC, the (−) enantiomer of 2',3'-dideoxy-3'-thiacytidine (3TC), and 2'-β-Fluoro-2',3'-dideoxycytidine, are presently undergoing clinical testing in patients with HIV infection. ddC is also being made available to patients in the United States who cannot tolerate AZT or who are failing AZT therapy under the regulatory mechanism of an Open Label Protocol.

analogues of dideoxypurines[39] are active compounds against HIV. These all may be candidates for clinical testing in the future.

The emergence of drug-resistant isolates can confound any therapeutic strategy. In this regard, the clinical response to AZT, the first of these compounds to be tested, was noted to be only transient in some patients; the CD4 count usually rose during the first several weeks, but then fell. It was subsequently found by Larder, Richman, and colleagues that patients on long-term AZT therapy frequently developed resistance to AZT.[40–42] Resistant strains generally had one or more mutations in the HIV *pol* gene (most frequently $Asp^{67} \rightarrow Asn$, $Lys^{70} \rightarrow Arg$, $Thr^{215} \rightarrow Phe$ or Tyr, or $Lys^{219} \rightarrow Gln$). In general, more than one base pair substitution was needed to yield a resistant phenotype, and this can explain the relatively slow development of resistance to this agent. Interestingly, these resistant strains preserved their sensitivity to

most other dideoxynucleosides (including ddC and ddI), although there was cross-reactive resistance to another 3'-azido-substituted nucleoside, 3'-azido-2',3'-dideoxyuridine (AzdU). The development of AZT resistance occurs at the time that CD4 counts generally start to decline. However, it has not yet been formally proven that there is a cause-and-effect relationship between these two events, and it is important that researchers continue to study this issue. Studies are now underway to determine how quickly resistance to ddC or ddI develops in a clinical setting. Our laboratory has failed to detect resistance in isolates obtained from patients receiving ddI therapy for up to 2 years.[43] However, two groups recently reported that some HIV isolates obtained from patients receiving ddI for several months had up to 10-fold reduced sensitivity to ddI.[44,45] Additional studies will be needed to determine the prevalence and clinical significance of ddI (and ddC) resistance. We would expect drug-resistant isolates to be identified in the future in at least a subset of patients receiving long-term therapy with these drugs.

As noted above, dideoxynucleosides act by chain termination of growing strands of DNA. Thus, it should not be surprising that each of these compounds tested to date has had some toxicity. Long-term toxicity is of particular concern in anti-retroviral therapy since this approach most likely requires treatment on a continuous basis, perhaps for the life of the patient. Certain of the toxicities of these drugs appear to be related to their inhibition (as 5'-triphosphates) of mitochondrial DNA polymerase (gamma polymerase). For example, patients on long-term AZT therapy frequently develop myositis associated with mitochondrial dysfunction.[46,47] The pathogenesis of other dideoxynucleoside-induced toxicities is less well understood, and preventing their development would certainly have clinical benefits. As will be discussed below, one approach to this is the use of combination regimens of dideoxynucleosides with different toxicity profiles (for example AZT and ddC or AZT and ddI). At the same time, the problem of toxicity from dideoxynucleosides has spurred interest in finding new classes of anti-HIV agents.

2.2. Non-nucleoside Reverse Transcriptase Inhibitors

During the past several years, several compounds other than nucleoside analogs have been identified as having potent and selective *in vitro* activity against HIV type 1 (HIV-1) reverse transcriptase.[48,49] The first of these exciting compounds reported were benzodiazepine analogues known as tetrahydro-imidazo[4,5,1,jk][1,4]-benzodiazepin-2(1H)-one and thione (TIBO) derivatives.[48] Several of these derivatives were found to have extremely high therapeutic indices *in vitro*. Interestingly, the compounds were highly specific for HIV-1; they had little or no activity against a variety of other retroviruses, including HIV-2. Other groups have since reported that certain compounds that shared certain structural features (for example, most have a seven-member ring with two nitrogen substitutions) had a similar pattern of specificity (including a lack of activity against HIV-2).[49] These compounds appear to inhibit reverse transcriptase by a mechanism that is non-competitive with respect to the primer, the template, the nucleotide, and tRNA. In addition, several members of this group have been found to have very little toxicity when tested in animals. These latter characteristics would appear to be favorable predictors of anti-HIV activity in patients.

Preliminary results from the first of these compounds to enter clinical testing (R 82913 TIBO derivative) indicated that the drug was well tolerated. Serum levels

well above the 50% inhibitory dose (ID_{50}) of 0.0015 μM were attained in patients. However, while some patients had decreases in HIV p24 antigen, there were no changes in CD4 counts, and overall, there was no clear-cut evidence of anti-HIV activity.[50] The dosing on this trial was limited by drug supply (this particular compound is very difficult to synthesize), and it is still unknown whether any of these drugs will in fact be found to have clinical activity. If they fail to yield clinical benefit as single agents or if the benefits are only transient, it will be of interest to see whether this is because of the development of viral resistance. Second or third generation congeners in this class could address these problems. It is possible that the ultimate role of this class of compounds will be for use together with a dideoxynucleoside such as AZT – combination therapy may slow the development of resistance to both agents.

3. COMPOUNDS THAT INHIBIT VIRAL BINDING

The initial step in the replication of HIV is its binding to the target cell. In most situations, this appears to be mediated by the binding of HIV gp120 Env protein to CD4 on the cell surface.[51,52] It has been found that this step can be inhibited by a variety of compounds, both in a non-specific and a specific manner.

For example, several polyanionic sulfated polysaccharides (including dextran sulfate and pentosan polysulfate) were found to have anti-HIV activity *in vitro*.[53–56] However, none of these compounds has been found to have activity when tested in patients with HIV infection. One of these compounds, pentosan polysulfate, was also of potential interest because it was found to inhibit basic fibroblast growth factor (bFGF) *in vitro*.[57] There is some evidence that bFGF potentiates the growth of Kaposi's sarcoma cells *in vitro*,[58] and it was hypothesized that bFGF might have some activity against Kaposi's sarcoma. Preliminary results from a trial of pentosan polysulfate conducted at the National Cancer Institute, however, did not reveal evidence of tumor shrinkage, although certain patients may have had a stabilization of their disease (J. Pluda, S. Broder, and R. Yarchoan, unpublished observation).

Why have these compounds not been found to have clinical activity? One possible explanation is that they may fail to adequately penetrate solid lymphoid organs. Also, they may be relatively inefficient at inhibiting cell-to-cell spread of HIV. Another possible reason is that such compounds are highly bound to protein,[59] and the concentration of free drug *in vivo* is almost certainly less than that in cultures enriched with 10% fetal calf serum (the usual conditions for *in vitro* study). Thus, several factors may explain the failure of these compounds to work in the clinic. However, the results with these drugs do serve as a reminder that the identification of activity of agents *in vitro* is not a guarantee of clinical activity.

More recently, several laboratories have produced soluble forms of CD4 by recombinant technology (rCD4), and these have been found to inhibit infection by T cells and monocytes by laboratory strains of HIV *in vitro* at concentrations of 1 to 5 μg/ml.[60–64] A theoretical advantage of this approach is that rCD4 could prevent the "bystander" killing of HIV-uninfected CD4+ T cells.[65] It has been shown that upon being exposed to free HIV gp120 (as may occur *in vivo*), such cells may be killed by gp120-specific cytotoxic T cells.[66] By binding to free gp120, rCD4 (or its analogues) might prevent this from occurring.

One question concerning this general approach was whether HIV could under certain circumstances enter cells via mechanisms that do not involve CD4, and if so,

whether this was of physiologic significance. Certain cell lines of neural or muscular origin do not express CD4, yet have been reported to be susceptible to infection by HIV, and infection of these lines is not inhibited by rCD4.[67] It has been shown that anti-HIV antibodies can enhance the infection of target cells;[68-70] under certain circumstances (e.g. in the case of cytomegalovirus-infected fibroblasts), entry appears to occur via Fc receptors, bypassing CD4.[71] Monocytes are important target cells for HIV infection and express Fc receptors, and we wondered if they might be infected by HIV via a CD4-independent mechanism in the presence of enhancing antibodies. However, we did not find this to be the case; even in the presence of such antibodies, infection of peripheral blood monocytes by HIV was completely inhibited by rCD4.[72] There is some evidence, however, that infection of certain monocytoid lines by HIV may in fact occur via a CD4-independent mechanism in the presence of enhancing antibodies,[69] and this issue will require further study to sort out.

Another potential problem with the use of rCD4 as anti-HIV therapy was that the serum half-life is rather short (of the order of an hour).[73-75] To address this problem, chimeric proteins combining the gp120-binding domain of CD4 with the constant part of IgG (rCD4-IgG) have been constructed. rCD4-IgG, which is sometimes referred to as an "immunoadhesin", was found to be similar to rCD4 in its inhibition of HIV infection *in vitro*.[76] Immunoadhesins were found to remain in the circulation for a longer period of time than rCD4, thus allowing higher levels to be attained. Phase I trials of both rCD4 and rCD4-IgG have shown these compounds to be well tolerated.[73-75,77] However, even at the highest levels tested, there was no clear cut evidence of anti-HIV activity.

One possible explanation for this observation is that the laboratory strains used for the initial testing are more sensitive than the strains existing in patients. Indeed, Daar, Ho, and colleagues have reported this to be the case; primary isolates of HIV were found to be substantially less sensitive to inhibition by rCD4 than HIV-IIIB or LAV (two commonly used laboratory strains).[78,79] Indeed, no substantial toxicity has been observed with rCD4-IgG in patients, and it is possible that doses higher than those employed in the initial trials would be found to have activity. In this regard, preliminary results suggest that relatively high doses of rCD4-IgG may induce increases in the platelet counts in patients with HIV-associated thrombocytopenic purpura.[80] It will be of interest to see if this is the result of an anti-HIV effect.

Ward and colleagues have recently shown that infection of chimpanzees by the IIIB laboratory strain of HIV could be prevented by pre-treatment with rCD-IgG.[81] This is an intriguing result, and it will be of interest to see if similar results in chimpanzees are attained with fresh isolates of HIV. It is conceivable that such agents may eventually find clinical utility in preventing primary infection with HIV or in preventing perinatal transmission.

4. AGENTS WHICH ACT AT OTHER STAGES OF HIV REPLICATION

4.1. Protease Inhibitors

A number of agents which work at other stages of HIV infection are now under development. As discussed elsewhere in this volume, one of the more exciting classes of anti-HIV agents now under study are the inhibitors of HIV aspartyl protease. The gag and pol proteins are originally translated as a large polyprotein (gag-pol fusion polyprotein) which must then be cleaved by viral protease (a *pol* gene product) in

of rationally-designed therapies, and agents which act at different steps are now under development. The advent of such therapies offers the possibility of developing regimens in which HIV replication is inhibited at multiple steps. Indeed, it is doubtful that any single agent will ultimately be found to be the optimal approach for the treatment of HIV infection.

There are a number of potential benefits of combination regimens. One is the reduction of toxicity. For example, toxicity profiles of the dideoxynucleosides AZT and ddC are markedly dissimilar; the dose-limiting toxicities of AZT are bone marrow suppression and myopathy, while that of ddC is painful peripheral neuropathy. Taking advantage of this, our group at the National Cancer Institute initiated a trial of alternating AZT and ddC therapy.[13] Preliminary results from this trial, which is still ongoing, suggest that some patients can have a sustained anti-HIV effect and that the toxicity from either drug may be reduced as compared to full-dose single agent therapy.[29] Other regimens exploring alternating AZT and ddC are now being tested by the AIDS Clinical Trials Group (ACTG) of the National Institute of Allergy and Infectious Diseases (NIAID).[30] Also, Pizzo and co-workers have found that an alternating AZT/ddC regimen can provide a sustained anti-HIV effect in children.[96] ddI also has a different toxicity profile to that of AZT, and combination studies of AZT and ddI are now underway in a number of centers.

Another potential benefit of combination therapy may be to prevent or delay the development of resistance. This may apply even to combinations of dideoxynucleosides, as HIV isolates resistant to AZT appear to preserve their sensitivity to other dideoxynucleosides. Indeed, it is possible that certain drugs which rapidly induce resistance if given as a single agent will have clinical utility only when used in combination with other drugs.

Certain combinations of anti-HIV agents appear to have synergistic anti-HIV activity *in vitro*,[53,56,97-99] and this may provide yet another rationale for combination therapy. One might expect synergistic interactions to occur particularly with agents that act at different steps of the HIV life cycle, and in fact this is one rationale for pursuing combinations of various types. However, certain drug combinations may antagonize each other. For example, ribavirin markedly reduces the anti-HIV activity of AZT *in vitro*[100] by inhibiting its phosphorylation. This observation serves as a warning against the ad hoc use of drug combinations without adequate *in vitro* testing.

It is possible that administration of drugs that suppress certain opportunistic infections may indirectly affect the level of HIV replication. Combinations of such drugs with anti-HIV agents may thus be worth exploring. Regulatory proteins produced by certain herpes viruses or adenovirus, for example, can transactivate HIV,[101] and suppression of these viruses might thus indirectly suppress HIV replication. In a similar vein, infection of CD8-bearing T lymphocytes by human herpes virus 6 has been shown to induce expression of CD4 on such cells and render them susceptible to infection by HIV.[102] It is conceivable that inhibition of human herpes virus 6 might thus help in the suppression of HIV suppression.

6. CONCLUSION

As we have seen, anti-HIV therapy has been shown to delay the progression to AIDS and to prolong the survival of patients with HIV infection. However, while we generally can induce increases in CD4 counts with such therapy, such gains are

generally transient with available drugs, and the progressive decline of CD4 counts then resumes. This does not have to be the case; it is quite possible that sustained improvements in the immune function in AIDS will be attainable in the not too distant future. In particular, while combination therapy has not been found formally to be superior to monotherapy in AIDS, it is likely that sustained responses will be most likely attained with a combination of agents.

While restoration of the immune system to its pre-morbid level remains an admirable goal, this may not be absolutely necessary, at least as a short-term goal. Indeed, a retrospective study of patients followed at the National Cancer Institute has shown that fatal opportunistic infections and tumors generally do not occur until the CD4 count falls below 50 cells/mm^3.[103] Thus, one can hypothesize that maintenance of the CD4 count above that level will yield a marked improvement in survival. It is quite conceivable that through the development of new agents and combination regimens, it will be possible to test this hypothesis in the not too distant future.

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From

The AIDS Reader®

Selecting Combination Therapy Using Data From in Vitro Studies CME

Author: Martin S. Hirsch, MD, Harvard Medical School, Massachusetts General Hospital, Boston, Mass.

Abstract: Combination therapy is the foundation upon which the hope of HIV eradication rests. Much of the information used to refine antiretroviral therapy was heralded by in vitro tests. This article reviews those lessons and the contributions made by these in vitro studies. [The AIDS Reader 7(4):116-119, 1997. © 1997 SCP Communications, Inc.]

Introduction

The concept of gaining control over a virus, with the hope of eventually eradicating it from the body, finds its basis in both oncology and infectious disease experiments started before World War II. During the past 50 years, steady advances led to multidrug combinations for eradicating diseases such as childhood leukemias and tuberculosis. The rationale for combination anti-HIV therapy includes expectations of:

- Additive or synergistic antiviral activity;
- Favorable pharmacokinetic interactions;
- Delayed emergence of drug resistance or broadened coverage against existing resistant virus; and
- Coverage of HIV in multiple cell types and tissues as well as cells at different stages of activation.[1,2]

Several questions regarding combination therapies remain unanswered, particularly those that relate to drug penetration of

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organs and tissues. Such questions may be difficult to address using in vitro techniques; nonetheless, when in vitro assays have been applied to potential antiretroviral regimens, they have predicted the results of subsequent clinical trials to a notable extent. Based on in vitro and in vivo experimentation, we have learned the following:

- Some combinations are synergistic, others are additive, and others are antagonistic.
- The effects of combinations may vary, depending on drug and virus concentrations.
- Simultaneous combinations are generally more effective than alternating combinations.
- Assuming favorable individual interactions, more drugs create a more durable suppression (4>3>2>1).
- Both single-target (ie, 2 or 3 reverse transcriptase inhibitors [RTIs]) and multiple-target (ie, RTIs + protease inhibitor [PI] + nonnucleoside reverse transcriptase inhibitor [NNRTI]) combinations create profound and sustained suppression, both in vitro and in vivo.
- Breakthrough virus may or may not show phenotypic or genotypic resistance.

Although both in vitro studies and clinical trials have been moving in the direction of using 2, 3, and, perhaps, 4 drugs in combination, these therapies must be selected with care, as favorable interactions may fail to occur, and undue toxicity may result.

Several mechanisms may potentiate the effects of single agents in antiretroviral combinations. First, combining 2 agents that act on a single viral target (ie, HIV reverse transcriptase) might exert selective pressure against the emergence of drug-resistant virus or force HIV in the direction of less pathogenic variants, a mechanism that has been proposed to contribute to the effects of zidovudine plus lamivudine.[3] Second, combining agents that are activated in different cell types (ie, zidovudine and didanosine)[4] might broaden antiretroviral coverage to include both activated and resting cells. Third, combining agents that act at different stages of the HIV replication cycle (ie, inhibition of virus production by protease inhibitors and of infection by RTIs) may also broaden antiretroviral coverage.

Single-target and Multi-target

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Favorable interactions have been seen with 2 nucleoside analogue RTI combinations including stavudine plus lamivudine, stavudine plus didanosine, zidovudine plus didanosine, and zidovudine plus zalcitabine. Each of these combinations was first demonstrated in vitro to synergistically inhibit HIV-1.[5,6,7] Within 5 or 6 years of the in vitro studies, these observations had been confirmed by clinical studies, such as ACTG 175 and Delta [8-10]; these studies showed that the combinations were superior to zidovudine monotherapy. It is not surprising that the protease inhibitor saquinavir demonstrated potent anti-HIV activity when tested in vitro, nor that it potentiated the antiviral effects of RTIs.[11] However, at the time of those studies, the favorable interaction resulting from an attack of HIV-encoded enzymes that act at 2 different points in the HIV life cycle had not yet been established.

Antagonism Between Antiviral Agents

In vitro antagonism has been noted among certain antiretrovirals. Ribavirin, for example, inhibits the activation of zidovudine, thereby antagonizing its anti-HIV effect.[12] The combination of zidovudine plus stavudine has also been found to be antagonistic when applied against certain isolates of HIV-1.[13] The mechanism appears to be competition for enzymes necessary to phosphorylate stavudine. Clinical trials data recently gathered from ACTG 290 suggest that there may also be in vivo antagonism. (Interim Review, ACTG 290, Executive Summary, Oct. 30, 1996.)

With other combinations, however, effects on intracellular deoxynucleosides may lead to the potentiation of an antiretroviral effect, as is seen in the combination of hydroxyurea plus didanosine, where the effect of didanosine is potentiated.[14,15] Again, this combination is thought to act similarly in the human body.[7]

Extensive in vivo work has tested the concept that increasing the number of antiretroviral components may overcome the HIV breakthrough. In 1994, Mazzulli and coworkers[16] reported that there was greater suppression of HIV and delay in breakthrough virus replication as the number of drugs was increased from 1 to 4 agents administered simultaneously.

Simultaneous Versus Alternating Treatment

Mazzulli and colleagues[16] also looked at the degree and duration of suppression from simultaneous administration of 4 drugs compared with the degree and duration of suppression from alternating administration of 2-drug combinations. This investigation was based on the unproved theory that alternating combinations may be less toxic and better able to suppress the emerging resistant strains. Simultaneous administration was more effective than alternating treatment (Fig. 1). Similar results were obtained by Yarchoan and associates[17] in a clinical trial that compared alternating versus simultaneous zidovudine plus didanosine therapy,[17] as well as by the executive summary of preliminary results from the large clinical endpoint trial, ACTG 193a.

Figure 1. (click here to zoom) Four drugs given simultaneously were more effective than same drugs given as alternating combinations of 2 drugs. Inhibition of HIV-1 p24 antigen production in peripheral blood mononuclear cells treated with zidovudine (ZDV), interferon (IFN), didanosine (ddI), and saquinavir (SQV) simultaneously (left) or ZDV + IFN alternating with ddI + SQV every 7 days (right). The IC corresponds to drug concentrations, with IC99 representing the highest and so forth down to the lowest concentration, IC50. Adapted from Antimicrob Agents Chemother (1994; 38:656-661), Copyright © 1994, American Society for Microbiology.[16]

Resistance

Monotherapy has been well established as one of the major causes of resistance. For instance, most individuals develop high-level rapid resistance after a fairly short time on zidovudine, and virtually all develop high-level resistance to lamivudine immediately. Although combination therapy achieves more substantial and more prolonged HIV suppression than was previously considered possible, there is increasing concern over the selection of HIV isolates with multiple resistance mutations. These mutations may interact in many ways to increase or reduce the viability of HIV itself and/or increase or reduce the susceptibility of HIV to other antiretroviral agents. Because these interactions may be impossible to predict based on genotype alone, in vitro screening may provide a practical means for evaluating therapeutic options and guiding treatment decisions for individual patients. Additionally, efforts are under way using several combination regimens in vitro to treat various drug-resistant HIV isolates.

Synergistic drug combinations may be able to overcome at least modest levels of resistance to 1 or more agents. In the simplest scenario, such as HIV mutations that are resistant to zidovudine, in vitro synergy has been shown for combinations of zidovudine plus didanosine and zidovudine plus saquinavir.[5,11] Interactions detected by in vitro assays may be more complex. As mentioned previously, the combination of zidovudine plus stavudine is antagonistic against some HIV-1 isolates, although more pronounced against zidovudine-resistant strains.[13] Antagonism in the presence of zidovudine resistance negates the antiviral effect of both zidovudine and stavudine, creating a dangerous absence of therapeutic coverage. In another in vitro example, saquinavir and zidovudine were additive or synergistic against zidovudine-sensitive and zidovudine-resistant HIV-1 isolates.[11] However, interactions between certain protease inhibitors (particularly saquinavir and indinavir), with or without zidovudine, have recently been tested against a variety of HIV clinical isolates (zidovudine-sensitive, zidovudine-resistant, and multidrug-resistant).[18,19] These in vitro studies demonstrated that low-level antagonism was common, which highlights the need for caution in combining various protease inhibitors.

Other Considerations and Future Directions

Although in vitro studies have succeeded in predicting many results that have subsequently been obtained in vivo, other favorable pharmacologic interactions have first been observed clinically and further characterized by in vitro studies. For example, coadministration of ritonavir and saquinavir resulted in pronounced elevation of plasma saquinavir levels due to inhibition of cytochrome P450 by ritonavir, with resultant inhibition of saquinavir metabolism.[20,21] The NNRTI delavirdine also inhibits cytochrome P450 enzymes. When administered concomitantly with protease inhibitors, delavirdine increased plasma levels of saquinavir about 5-fold, increased levels of indinavir about 2-fold, and had no effect on ritonavir.[22]

In retrospect, many questions that ultimately have been answered in clinical trials have been foreshadowed by in vitro testing; had there been reason to develop appropriate in vitro models, other answers might also have been predicted. The questions arising from combination therapy and possible eradication of HIV have already been posed:

- Can HIV eradication be achieved in an infected individual?

- Can potent induction regimens be followed by simpler, less toxic, and less costly regimens?
- When is the best time to include protease inhibitors and NNRTIs in combination regimens?
- What combinations are

  indicated when drug-resistant viruses emerge?
- Can cycling of combinations reduce such emergence?
- Can ex vivo outgrowth inhibition assays be used to predict the success of combination therapies?

In vitro models to provide potential answers to these important questions are needed. Several of these strategies are being explored in vitro or ex vivo. As a case in point, my colleagues and I have used an ex vivo assay to establish the feasibility of CD4+ T-lymphocyte expansion and virus eradication in persons with HIV disease.[23] It is hoped that this type of assay may ultimately help predict which patients can safely have drugs withdrawn after prolonged therapy with antiretroviral combinations.

About the Author

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The Use of *In Vitro* Systems for Evaluating Haematotoxicity

The Report and Recommendations of ECVAM Workshop 14[1,2]

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Pr face

This is the report of the fourteenth of a series of workshops

organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

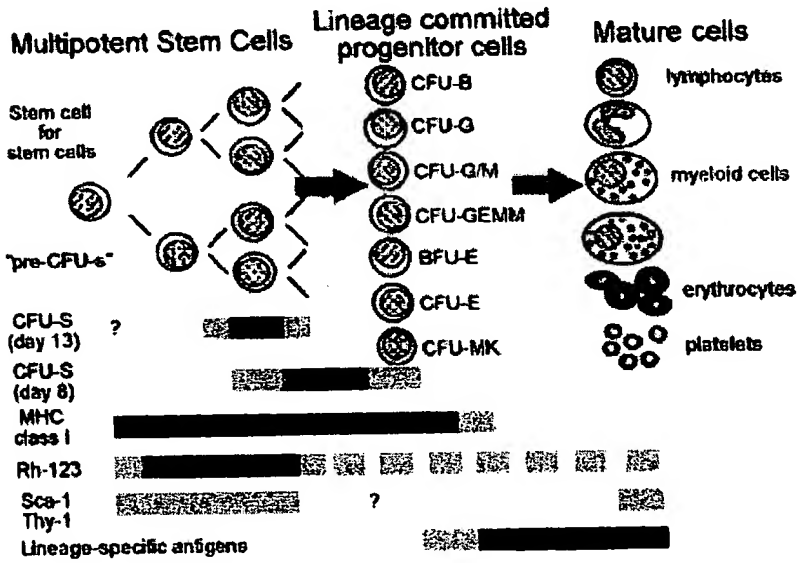
The workshop on The Use of *In Vitro* Systems for Evaluating Haematotoxicity was held in Angera, Italy, on 29 May-2 June 1995, under the co-chairmanship of Greet Schoeters (Flemish Institute for Technological Research, Mol, Belgium) and Augusto Pessina (University of Milan, Italy). The participants represented academia, national organisations, international regulatory bodies and industry. The aims of the workshop were to prepare a state-of-the-art review on *in vitro* haematotoxicology, including consideration of quantitative *in vitro/in vivo* correlations and interspecies differences, and to make recommendations about what further steps should be taken in terms of the development, prevalidation or formal validation of relevant and reliable procedures which could reduce the use of animals in biomedical research and toxicity testing in this particular area. These recommendations are outlined at the end of this report.

Introduction

Haemopoiesis (blood cell production) is a very complex process involving the interaction of many cell types and both local and systemic growth factors (2, 3). With respect to blood cell formation, two functional compartments must be considered: a) the haemopoietic stem and progenitor cells; and b) the stromal cells which constitute the haemopoietic microenvironment. The integrity of, and close cellular association between, these two compartments are essential for maintaining normal haemopoiesis (4, 5). Both the haemopoietic and the stromal cell systems are composed of many cell types and

are organised hierarchically (6, 7; Figure 1). A limited number of haemopoietic stem cells, which ar characterised by a multi-potential and self-renewing capacity, gives rise to progenitors of different lineages. These committed progenitors proliferate rapidly to increase tissue mass, and then they differentiate into mature blood cells in response to humoral growth factors and local cytokines in their particular microenvironment (8). Progenitors can be stimulated to respond similarly *in vitro*; this capacity is the basis underlying the widespread use of *in vitro* colony-forming assays (9, 10), in which the progenitors give rise to phenotypically distinct colonies of differentiated cells in the presence of tissue culture medium containing a specified mixture of cytokines for a particular haematological lineage.

Figure 1: Schematic Diagram Illustrating the Major Colony-Forming Assays and the Ontogenic Relationships between the Different Lineages



B = B-cell; BFU = burst-forming unit; CFU = colony-forming unit; E = erythroid; G = granulocyte; GEMM = granulocyte/erythroid/monocyte/megakaryocyte; MHC = major histocompatibility complex; G/M = granulocyte/macrophage; MK = megakaryocyte; S= spleen.

Taken from reference 7.

Due to its rapid turnover, haemopoietic tissue has the capacity to respond quickly and effectively to an increased demand for mature cells (for example, following blood loss

or infection), and can maintain this response for prolonged periods of time. This rapid rate of renewal also makes the haemopoietic system a major target for xenobiotic toxicity (11-13). Xenobiotics may interfere with its proliferative activity and with the complex regulation pathways.

The focus of this workshop was toxicity to the blood-forming elements. However, toxicants can also cause haematotoxicity by interfering with mature blood cell function or survival, or by interfering with lymphocyte production and function. The latter topics were considered to be outside the scope of this workshop, since such toxic effects result in very different clinical symptoms, and they require a very different *in vitro* approach for evaluation. We recommend that these be addressed in future workshops. Moreover, following cytotoxic insult, myelosuppression is generally considered to be a greater clinical risk than lymphocytopenia. This is based on the much more severe profile of clinical side effects of neutropenia and thrombocytopenia observed following chemotherapy. As a consequence of this, haemopoiesis is often the limiting factor when designing multi-drug chemotherapeutic or radiotherapeutic regimens (14).

This workshop was, to the knowledge of the participants, the first structured attempt to report on the state-of-the-art of *in vitro* methodologies for haematotoxicity testing, and to recommend initiatives which might help to bring these methods closer to validation and implementation. The workshop participants believe that data from *in vitro* haematotoxicology studies can aid in risk assessment procedures to refine human safety margins, while simultaneously reducing the number of animals used in preclinical toxicology studies.

Haematotoxicity *In Vivo*

Haematotoxicity can be primary or secondary, depending upon whether the haemopoietic system is affected directly or as a consequence of toxicity to other organ systems, respectively. In addition, primary haematotoxicity can be specific (that is, affecting only blood production) or non-specific (when other rapidly dividing organ systems, such as the intestinal tract and the gonads, are also affected). Haematotoxicity can be either acute or chronic, and can be reversible or irreversible following removal of the toxicant.

While severe haematotoxicity seen in cancer patients treated with cytoreductive agents is accepted as being

necessary to obtain the required clinical benefits, its occurrence as an effect of therapy for other diseases is clearly unacceptable. Given the seriousness of unexpected haematotoxicity encountered in the clinical setting, every effort must be made to develop screening tools which will reliably and reproducibly predict chemical-induced myelosuppression.

Currently, laboratory animals are used widely to predict haematotoxicity; *in vivo* toxicology studies in at least two animal species are required for the regulatory approval of food additives, industrial chemicals, and pharmaceuticals (15). Haematological parameters, such as peripheral blood counts, are monitored routinely in these animal studies, and are then used to predict likely human haematotoxic effects. The most commonly used species are rats, mice, and dogs. Rodents are used because of the ease of handling and the availability of extensive information on their haemopoietic system. The dog has been used because it offers several advantages over other animal species (16, 17), including: a) its haematological similarity to humans (for example, with respect to blood cell kinetics, the high granulocyte proportion of total leucocyte counts, and the anatomical ultrastructure of the bone marrow); b) the availability of data from experimental haematology (for example, information on bone marrow transplantation); and c) its size enables serial monitoring of blood cells and bone marrow cytology.

For the preclinical development of antineoplastics, *in vivo* toxicology studies are generally conducted in at least two different species, employing various dosing schedules. These *in vivo* data are used to derive the dose for the first human exposure, which is usually ten-fold or more below the maximum tolerated dose (MTD) in the animals. In the case of food additives and industrial chemicals, chronic toxicity studies are usually undertaken in rats, in which haematological parameters, such as peripheral blood counts, are followed routinely. The maximum permissible exposure limit (PEL) in humans is based on the concentration of the chemical at which no adverse effects are observed, generally after reducing this concentration by an additional safety factor of 100. Any *in vitro* test which can refine these safety margins, by reducing the toxicological uncertainties underlying laboratory animal/human extrapolations, would be of great benefit, since it would provide a more scientific and rational basis for calculating clinical dosages and for setting human exposure limits.

Haematotoxicity *In Vitro*

In vitro methods used in haematotoxicology fall into three categories: a) assays for the study of haemopoietic progenitors which are committed to one or more lineages; b) assays for the study of stem cells, which are primitive pluripotent cells with self-renewal and long-term repopulating capacities *in vivo*; and c) assays for the evaluation of toxic effects on the haemopoietic microenvironment.

Cell Sources

Primary cells are mainly used to initiate haemopoietic cultures. These proliferate and differentiate into various haemopoietic lineages. The cell cultures can be exposed to different toxicant concentrations and conditions. Recently, haemopoietic and stromal cell lines have been obtained which exhibit some properties of precursor cells (18-23). If these lines are well characterised pharmacologically and prove to be stable, they could be promising tools for future haematotoxicity testing, reducing further the use of experimental animals.

Bone marrow cells are mainly used to initiate haemopoietic cell cultures, but peripheral blood cells, spleen cells, and fetal haemopoietic tissues from a variety of animal species, including humans, can also be used. The most commonly used tissues are those from animals which are used routinely in the preclinical development of new compounds: mice, rats, dogs, and non-human primates. Human haemopoietic cells can be obtained from at least four sources: a) bone marrow from sternal aspiration when a myelogram is performed; b) femoral aspiration from patients undergoing orthopaedic surgery (24); c) bone marrow aspirates from the iliac crest; and d) umbilical cord blood samples collected from placentas after normal deliveries (25).

Progenitor Assays

Haemopoietic progenitors are already committed to one or more lineages of mature blood cells (9). Primarily, colony-forming assays are used to investigate the direct effects of toxicants on the proliferative capacities of the progenitors in semi-solid medium. These assays usually take 14 days or less. The progenitor assays include lineage-restricted, colony-forming units of: a) the erythroid lineage (colony-

forming unit-erythroid [CFU-E], and the less mature burst-forming unit-erythroid [BFU-E]); b) the myeloid lineage (colony-forming unit-granulocyte/ macrophage [CFU-GM]); and c) the megakaryocyte lineage (colony-forming unit-megakaryocyte [CFU-MK], and the less mature burst-forming unit-megakaryocyte [BFU-MK]; 26). There are also multi-potential, colony-forming units which, by definition, produce cells of multiple lineages (colony-forming unit-granulocyte/ erythroid/monocyte/megakaryocyte [CFU-GEMM]); these were discovered originally in the mouse (27) and were found subsequently in humans (28). One of the major advantages that the *in vitro* colony-forming assays offer is the possibility of employing bone marrow cells from humans to investigate comparative haematotoxicology across species. These assays can also be miniaturised by using micro-capillary culture tubes when there is a very limited supply of the test material (29).

Stem Cell Assays

Primitive stem cells are those cells which have long-term repopulating capacities, in contrast to progenitor cells, which have more limited proliferation potentials (30). *In vivo,* stem cells can be used to successfully treat lethally irradiated mice, resulting in their long-term survival and the development of all haemopoietic lineages (31, 32). The search for an *in vitro* assay employing pluripotent haemopoietic stem cells with properties of self-renewal and long-term repopulation is still ongoing (33). The enormous proliferative potential of primitive haemopoietic cells is evident in several *in vitro* assays, such as the assay for high proliferative, potential colony-forming cells (HPP-CFCs; 34, 35), the multi-potential stem cell colony-forming unit *in vitro* (CFU-A) assay (36), and an assay for quantifying blast cell colonies (37). These assays enable the identification of a quiescent haemopoietic cell population which is considered to be primitive.

A different approach is the sustained growth *in vitro* of primitive haemopoietic cells which are capable of generating haemopoietic progenitor cells in long-term liquid cultures, either in direct association with functional stromata (38) or on synthetic matrix supports (39). The sustained growth and differentiation of the stem cells *in vitro* depends upon the presence of an appropriate microenvironment, such as that provided by fibroblast-like stromal cells. The capacity of haemopoietic stem cells to generate "cobblestone" areas of haemopoietic cells and give rise to progenitors in pre-established stromata (40), has enabled quantitative studies on the stem cells to be

undertaken *in vitro.*

The use of purified cell populations in haematotoxicity studies enables the direct interaction of a compound with the haemopoietic progenitor population to be investigated. The enrichment or purification of different types of haemopoietic precursors can now be achieved by using positive and negative selection procedures based on: a) physical properties of the cells (light scattering properties, such as size and density); b) expression of cell surface markers which can be detected with monoclonal antibodies and lectins; c) cell cycle status; and d) the metabolic activity of the cell. Cell populations have been enriched with different progenitors, and even with repopulating stem cells, by using gradient sedimentation, counterflow centrifugal elutriation, immunomagnetic cell sorting, and fluorescence-activated cell sorting (41).

Despite the vast number of markers available, it is not yet possible to isolate haemopoietic precursors on the basis of the expression of a single specific marker. However, selection procedures based on combining the negative selection of cells which express markers of differentiating cell populations (generally referred to as "LIN-selection") with the positive selection of cells which express markers preferentially present in primitive haemopoietic populations (that is, CD34 in humans, and Ly6A/E or WGA in mice), has enabled considerable enrichment of primitive repopulating cells isolated from primary haemopoietic samples (42, 43). In addition, by using metabolic markers (for example, Rh-123) it is possible to fractionate the purified populations into short-term and long-term repopulating cells (44, 45). The non-responsiveness of primitive haemopoietic precursors to particular combinations of growth factors has enabled the enrichment of human stem cells, by culturing bone marrow grafts with the Kit ligand and interleukin (IL)-3 in the presence of 5-fluorouracil (46).

Stromal Cell Assays

In vivo, stromal cells provide the haemopoietic microenvironment. They are quiescent under steady-state conditions (47), but can proliferate in response to a particular toxic insult. The manner in which chemicals affect the proliferative capacity of stromal progenitor cells can be investigated *in vitro* by determining their effects upon the capacity of stromal progenitors to form colonies of adherent fibroblast-like cells (that is, colony-forming units of fibroblasts [CFU-F]; 48).

The haemopoietic stroma provides the functional microenvironment (for example, extracellular matrix, growth factors) necessary for the growth and differentiation of the haemopoietic cells. Several toxicants are capable of producing haemopoietic dysfunctions by disturbing the functional activity of the stroma (49). In this respect, the haemopoietic re-feeding of chemical-treated stromata established in Dexter-type (38) or three-dimensional (50) cultures will facilitate an understanding of the role of the stroma in haematotoxicity. Also of significance is the fact that stromal cells have been shown to have cytochrome P450 (CYP450)-mediated metabolic activities and other bioactivation capabilities (51-55).

Current Use of In Vitro Assays

At present, *in vitro* colony-forming assays are used as screens, as investigative tools, and in the clinical setting of bone marrow transplantation. They can be used to screen for new drug leads of low haematotoxic potential (for example, for new antineoplastics or antivirals [56]), and to aid in the risk assessment of pesticides, industrial chemicals, etc. (57, 58). These assays can also be used to select the most appropriate animal species for use in the preclinical evaluation of a compound (59). In addition, the assays are used for therapeutic index-based screening to identify less-myelosuppressive analogues, for deriving structure-toxicity relationships, and for identifying any potential synergistic effects of compounds which may be administered simultaneously to patients (chemotherapy cocktails; 60).

As investigative tools, *in vitro* assays are used for: a) studying mechanisms of haematotoxicity of radiation, chemicals, and biological agents; b) identifying target sub-populations of cells in the haemopoietic tissue (stem cells, progenitor cells, or the microenvironment); and c) characterising the nature of effects on the target cell and predicting the reversibility of these effects (61-63). *In vitro* haematotoxicity assays can be used to detect lesions in immature haemopoietic compartments which cannot be detected by routine bone marrow cytology and histopathology until the effects are at advanced stages. Although clonogenic assays have been used successfully for studying the mechanism of chemical-induced acute, reversible, neutropenia, at present there are a lack of data indicating which *in vitro* targets and endpoints should be used to predict other types of haematotoxicity.

The predictivity of *in vitro* data has been shown in validation studies with antineoplastic agents and several anti-human immunodeficiency virus (anti-HIV) agents (64). Colony-forming assays have also been used retrospectively for studying drug-induced agranulocytosis, or aplastic anemia (65-75). Although this is a more difficult application, because of the sporadic nature of the adverse event, bone marrow cells taken from patients after they have recovered have been cultured in the presence of the drug and/or serum from the drug-treated patients to determine whether the patients are extremely sensitive to either the parent drug or a metabolite. Alternatively, drug-containing plasma samples from affected individuals can be tested on normal human marrow cells to determine whether the aplasia is caused by exposure to a haematotoxic metabolite which is only generated in a small, polymorphic group of people. Environmental haematotoxic compounds, such as mycotoxins, can also be tested in retrospective studies, to confirm their roles in causing various haematological disorders (76).

As a research tool, the *in vitro* assays can be used to study the modulation of drug-induced haematotoxic effects by various cytokines and growth factors. Cytokines are increasingly being used clinically. The introduction of myeloid growth factors, such as granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-3, IL-6, and stem cell factor, has provided haematologists and oncologists with possible agents to shorten cytopenias and resolve infections, and which could enable chemotherapy to be administered without delay (77). Colony-forming assays have contributed to elucidating the biological functions of various cytokines; progenitors are direct targets for several of these molecules (78-80). Haematotoxicity can result from interference with cytokine production, cytokine receptor binding, presentation at the cell surface, and/or secretion. In addition to cytokines, growth factors, hormones, the extracellular matrix, and other substances produced by cells in the microenvironment, could be involved in xenobiotic-mediated toxicity, but there is little information available about this. The use of *in vitro* assays has also contributed to the elucidation of lineage inter-relationships between various haemopoietic progenitors.

For clinical applications, such as bone marrow transplantation, *in vitro* assays are used to determine the number of progenitors in the potential graft. This information is needed to calculate the number of cells to

be grafted. Transplantations of this type are being used increasingly for the treatment of both haemopoietic and non-haemopoietic diseases (for example, for treating solid tumours [81]). The therapeutic procedures employed currently require manipulation of the haemopoietic grafts, which frequently involves the mobilisation of bone marrow progenitors to peripheral blood (achieved by using cytotoxic drugs and/or haemopoietic growth factors), freezing of the haemopoietic sample, and the positive or negative selection of particular cell populations by using immunomagnetic procedures. The expansion of haemopoietic progenitors *in vitro* prior to returning them to the patient is considered to be a new alternative for accelerating haemopoietic recovery following transplantation (82, 83). In addition, in numerous gene therapy and gene-marking protocols, *in vitro* manipulation of the haemopoietic graft is needed to facilitate the transfection of the haemopoietic stem cells with viral vectors bearing the therapeutic or reporter gene (84-92). Most of these processes require the labelling of the haemopoietic progenitors with monoclonal antibodies, and the activation of the stem and progenitor cells with complex combinations of growth factors and media supplements, whose deleterious effects on the longevities and differentiation potentials of the stem cells need to be determined. Cell culture assays will be useful for evaluating the potential haematotoxic effects which may result from the expression of, or even the simple insertion of, foreign genes in haemopoietic cells by biological or physical procedures.

During the planning of clinical trials, *in vitro* assays can be used to: a) identify the schedule dependency of myelotoxic effects (which, in turn, provides information about possible mechanisms of action); b) plan cytokine support; and c) estimate dose-escalation effects.

In Vitro/In Vivo Correlations: the Pharmacological Basis for the Design of *In Vitro* Experiments and the Interpretation of *In Vitro* Data

There is now ample evidence in the literature to demonstrate the close agreement between *in vitro* and *in vivo* haematotoxicity data. Ionising radiation, haematotoxic viruses, and several classes of directly haematotoxic compounds (including 3'-azido-2',3'-dideoxythymidine [AZT] and other antivirals, trichothecene mycotoxins, pesticides, benzene metabolites, methotrexate, 1,3-bis-(2-chloroethyl)-1-nitroso-urea [BCNU], and arabinoside C),

have been evaluated *in vitro* by several laboratories, and the *in vitro* data show good inter-laboratory reproducibility and agree well with the *in vivo* data (11, 12, 15, 57). For all of these compounds, there was considerable agreement between the active range of concentrations *in vitro* and *in vivo*, despite the fact that the methodology had not been optimised for undertaking such comparisons.

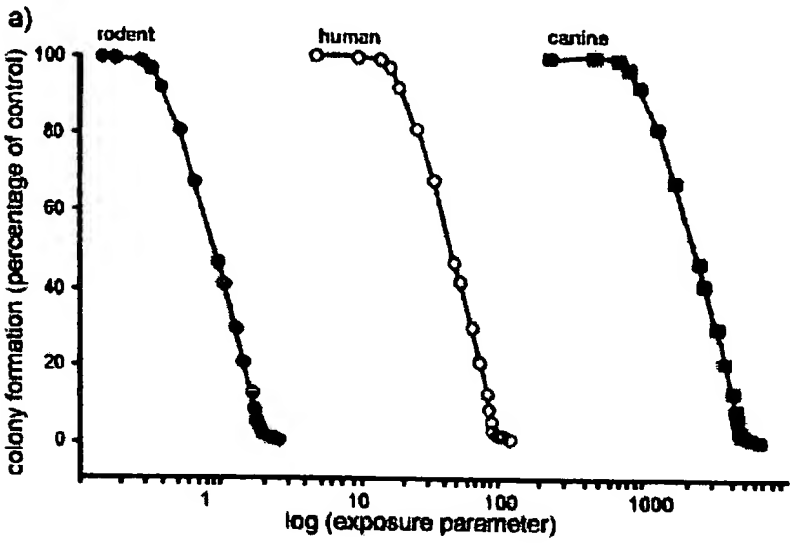
The severity of haematotoxicity depends upon the level of toxicant exposure; this is defined by pharmacological parameters describing the concentration of the toxicant in the plasma and the microenvironment, which include the threshold concentration, levels of haematotoxic metabolites, drug binding by plasma proteins, and the duration of exposure (determined from the clearance *in vivo* and from the chemical stability *in vitro*). The pharmacological relationship between toxicant exposure and haematotoxicity provides the basis for extrapolating *in vitro* data to the *in vivo* situation, thereby enabling quantitative predictions of the severity and nature of clinical haematotoxicity to be made.

Toxicant Exposure

In vitro data must be obtained which span the entire range of toxicity, from no significant effect to complete inhibition of the *in vitro* endpoint selected, because the plasma concentration in humans is not known at the time of the *in vitro* analysis, or during the extrapolation to humans of results obtained from preclinical animal studies. A complete analysis provides concentration-response curves, which may differ between species.

It is important to decide upon the most appropriate *in vitro* endpoints to be used for inter-species comparisons of relative toxicity (the inhibitory concentration [IC]). This depends upon the nature of the *in vitro* data and the anticipated and acceptable severity of haematotoxicity in humans. A particular compound may exhibit parallel curves across species *in vitro*, and in this situation inter-species comparisons are based on IC50 values (Figure 2A). Parallel curves from the various test species indicate similar, if not identical, mechanisms of action and target cell population(s) (and bioconversion, if metabolism occurs *in vitro*). In this case, the ratios of the IC values obtained for different species are identical when compared at any degree of inhibition between 5-95%; thus, quantitative comparisons of the potency of the toxicant across the species can be based on ratios of the IC50 values, since this is the most accurate value from a sigmoidal curve.

Figure 2: Theoretical Curves of *In Vitro* Data which Relate the Inhibition of an Endpoint which Predicts Neutropenia to the Level of Exposure to the Test Compound

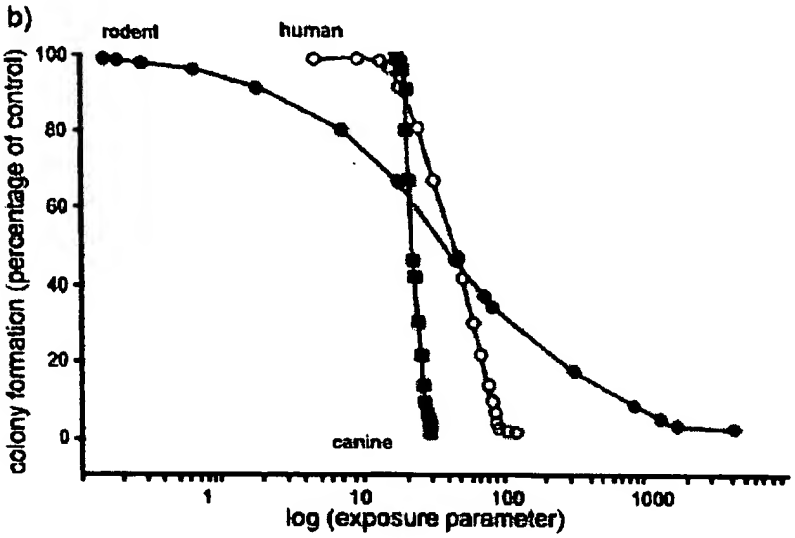


a) The shape of the exposure-response curves are identical for the three species; this is an "ideal" situation in which interspecies comparisons of xenobiotic sensitivity should be based on IC50 values.

A more common situation is that in which non-parallel curves are obtained for the different species (Figure 2B). In this case, knowledge about the desirable or acceptable level of haematotoxicity *in vivo* is needed for interpreting the *in vitro* results; the conclusions derived from the *in vitro* data are application-dependent. For example, if the data in Figure 2B are for an antineoplastic compound, then inter-species comparisons might be undertaken with the IC90 values, since a comparative study has related the IC90 value to grade 3-4 neutropenia (93), and most cytotoxic antineoplastics are administered up to this maximum tolerated level of myelosuppression. In contrast, if the compound is a potential environmental contaminant or a food additive, which regulations require to be present at levels well below the highest non-toxic levels, or a therapeutic agent to be given daily over a long period of time (for example, antivirals), then the comparison should be made at levels of inhibition which are known to be tolerated *in vivo* without the appearance of clinical symptoms. Since the minimum number of progenitors required to maintain normal peripheral blood cell counts *in vivo* has not yet been determined for any haematological

lineage in any species, the no-observable effect level (NOEL; that is, the IC0) should be used at present for food additives and contaminants, potential environmental contaminants, and all non-toxic therapeutics.

Figure 2: continued



b) The curves are qualitatively and quantitatively different, as in most "real-life" situations. In this case, the IC values used for interspecies comparisons will depend upon the intended use of, or exposure to, the compound (see text).

This philosophy of data interpretation, which is based upon the pharmacological context and intention of xenobiotic use/exposure in humans, dictates that the generation of data *in vitro* should be weighted toward that part of the concentration-response curve which will be used for inter-species comparisons, in order to obtain the most accurate determinations of the IC values which are appropriate to the particular situation.

Most antineoplastics exhibit area-under-the-curve (AUC)-dependent haematotoxicity in humans (94), although there are exceptions to this, such as dependency on the length of time spent above a threshold concentration, Cpmax (95), and cases in which haematotoxicity is not dose-limiting. However, antineoplastics generally have very well characterised haematotoxicity profiles, produced as a result of routine pharmacokinetic haematology correlates during dose escalation in Phase I clinical trials. With these compounds, pharmacological validation of *in vitro* assays can be achieved by showing that the pharmacodynamic

relationship between exposure and haematotoxicity is identical *in vitro* and *in vivo*.

With other classes of compounds, the relationships between plasma drug concentrations and haematotoxicity are not usually so well defined, either because clinical trials are designed specifically to avoid haematotoxicity, or because of ethical concerns regarding the administration of the compound to humans up to the MTD. The *in vitro* assays provide an opportunity to obtain values for the particular pharmacodynamic parameter which determines the severity of myelosuppression in humans, without having to reach this level clinically.

Duration of Exposure

In vitro data should be obtained not only for a range of concentrations, but also for a range of durations of exposure. For validation purposes, the question is whether the pharmacodynamic parameter which determines the inhibition of haemopoiesis *in vitro* is the same as that *in vivo*, and whether the duration of exposure contibutes to the inhibition observed. Data from *in vitro* studies have been published for the schedule-dependent, cell cycle phase-specific antineoplastics, methotrexate and topotecan (11); the compounds showed greater toxicity when the exposure was prolonged, even though this is based on the AUC. This is consistent with the known schedule dependency of myelosuppression *in vivo*, in which continuous infusion or multi-day dosing schedules produce significantly worse cytopenias (96-98). However, if the anticipated clinical dosing schedule is multi-day or prolonged continuous infusion, then brief exposures *in vitro* are unnecessary. In addition, if drug stability data indicate rapid decomposition *in vitro* (for example, with alkylating agents like BCNU), then only brief exposures *in vitro* are required; in fact, prolonged exposures with unstable compounds may be misleading, because the breakdown products might be myelotoxic, but this would be irrelevant if they are not actually formed *in vivo*.

Role of Added Growth Factors

Laboratories vary with respect to the protocol used for the addition of colony-stimulating factors (CSFs) to CFU-GM cultures during brief exposures to test compounds, prior to plating the cells in the absence of the compound. For *in vitro* tests with short exposure periods, it may be important to standardise upon the time which should elapse after the addition of CSFs before test compounds are added,

especially if the effects of the compound are known to be cell cycle phase-specific. CSFs also inhibit the apoptosis of progenitors (99, 100), and it is important to consider whether the high levels of CSFs *in vitro* may be partially, and artifactually, protecting the progenitors from being killed. Pharmacological levels of CSFs are probably not present *in vivo* during drug exposure, unless the microenvironment itself is able to maintain high local levels. The conditions of drug exposure *in vitro* should reflect as closely as possible the conditions in the bone marrow microenvironment *in vivo.*

Metabolites

While many compounds are metabolised to a variety of inactive chemical species, some compounds undergo bioactivation. Indeed, this is a prerequisite for those compounds which are administered as pro-drugs. Two compounds which are activated by metabolism are cyclophosphamide and benzene. To obtain predictive *in vitro* data for such compounds, a metabolising system (CYP450 in these cases) must be incorporated into the *in vitro* test system or testing strategy. Hepatocytes can be co-cultured with the haemopoietic cells, but as a physically separate cell layer. The toxicity of cyclophosphamide to progenitors *in vitro* was markedly increased when hepatocytes were present, which is consistent with *in vivo* data. Nevertheless, a small amount of bioactivation of cyclophosphamide and benzene occurs in the presence of bone marrow stromal cells alone (in the absence of hepatocytes). This is consistent with previous reports that bone marrow stromata contain drug metabolising enzymes, such as CYP450-mediated activities, although the specific activities are much lower than in the liver (50-55). Thus, future work needs to focus on a detailed characterisation of the capacity of the bone marrow microenvironment to metabolise xenobiotics.

For new compounds, metabolites should be generated (for example, by using hepatic microsomes, liver slices or hepatocytes), and tested both in the presence and absence of the parent compound itself, to identify the possible contribution of a metabolite(s) to the haematotoxicity observed, and to determine whether there are any synergistic or antagonistic effects between the metabolite(s) and the parent compound which might affect haematotoxicity *in vivo.*

S9 fractions and liver microsomes obtained from dogs and rats are commonly used to generate metabolites *in vitro.*

How ver, it can b very misleading to use these preparations without consideration of species-specific metabolism. For example, iododoxorubicin is metabolised in humans to the 13-hydroxy derivative, which contributes to clinical toxicity; thus, exposure to this metabolite must be included in determinations of toxicant exposure levels (101). This species-specific contribution of the metabolite to toxicity does not occur in the animal species used in preclinical studies. These data indicate that species-specific sources of metabolising enzymes should be used (human microsomes with human bone marrow, rat microsomes with rat marrow, etc.) for subsequent extrapolation of the *in vitro* data to the *in vivo* situation.

Free (Unbound) Xenobiotic Concentrations

Another species-specific effect which could result in erroneous conclusions being drawn from the *in vitro* data is the difference in the binding of xenobiotics by plasma proteins such as albumin. This is particularly significant for compounds which bind tightly to carrier proteins, and species differences in protein binding become more important as the affinity of the compound for the carrier protein increases. Most *in vitro* assays utilise horse serum or fetal bovine serum (FBS) at a concentration of 10-30% (v/v). Obviously, neither of these sera are relevant to the *in vivo* situation.

Since species-specific drug binding phenomena can be measured in other, relatively simple, *in vitro* systems, it may not be necessary to incorporate the particular binding proteins into the *in vitro* haematotoxicity assays *per se*. In fact, it may be advantageous to compare the sensitivities of different species to the toxicant under identical culture conditions, to learn about intrinsic differences in target organ sensitivities, and then to correct the *in vitro* data for differences in protein binding which have been determined by using other assays. In addition, it could be that development work should be supported for assays which use homologous plasma rather than FBS or horse serum.

To facilitate the extrapolation of *in vitro* data to the *in vivo* situation, the concentration-inhibition curve should be presented as the percentage inhibition of the endpoint versus the free concentration of xenobiotic soluble in the medium, rather than versus the total concentration of the xenobiotic. From this curve, plus measurements of free versus total xenobiotic concentrations in human and animal plasma, it should be possible to make pharmacodynamic-based correlations for all species. Data

obtained with anthracyclines (presented at the workshop), and with camptothecins (102-104), indicate that it cannot be assumed that chemically-related compounds will have identical protein binding and stability *in vitro* or *in vivo*, nor can it be assumed that a particular compound will behave identically in plasma from different species.

Age

Age is an important consideration if the toxic insult may occur, for example, during fetal development. The sensitivities of the haemopoietic cells and their micro-environment could be different for the developing individual and the adult. Age-dependent differences in the generation of long-term haemopoietic dysfunctions have been described previously, following the exposure of mice to radionuclides and external irradiation (105, 106). Stromal failure is considered to play an essential part in the generation of these effects (107).

In vitro studies have revealed differences between the responses of murine long-term cell cultures initiated from haemopoietic tissue taken from mice of different ages. The stromal micro-environment seemed to be responsible for these age-related differences in haemopoietic activity (108).

The Future of Haematotoxicity Testing *In Vitro*: Perspectives on Validation

The objectives of toxicology studies are: a) the identification of potentially dangerous toxicants, so that human exposure can be prevented/controlled; and b) the provision of information relevant for undertaking risk-benefit analyses and for conducting clinical trials. *In vitro* haematotoxicity assays which have been validated (they are predictive for *in vivo* effects, understood with regard to their clinical relevance, and their limitations are known) could play a significant role in forming a bridge between preclinical animal toxicology studies and: a) the clinical development of new therapeutic agents (11-13, 109); and b) human risk assessment of food additives and potential environmental contaminants.

Validation is the process by which the reliability and relevance of a procedure are established for a specific purpose (110). The reliability of a procedure describes whether it can be performed reproducibly within and among laboratories and over time. Reproducibility is

achieved by optimising the conditions for each assay, and by establishing consistency in the procedures used for sampling, cell preparation, and scoring the test endpoints. The reproducibilities of the *in vitro* colony-forming assays need to be established, prior to attempts to establish th ir relevance as predictive tools for clinical haematotoxicity, and before *in vitro/in vivo* comparisons are undertaken. Only after the methodology has been validated will it be possible to determine whether *in vitro* haematotoxicity assays provide meaningful data of benefit for regulatory purposes. Formal validation of the human CFU-GM assay is needed, both of the methodology itself and of the predictivity of the assay results for clinical neutropenia. It is envisaged that, once validated, the CFU-GM assay could be included in the routine battery of toxicological test methods and could contribute to regulatory decisions, while at the same time leading to the refinement of any subsequent animal tests, which are considered to be necessary, and to a reduction in the number of animals used.

Due to the complexity of *in vitro/in vivo* relationships, it would appear that the most important next step is to undertake *in vitro/in vivo* comparisons for a number of cytotoxic compounds (antineoplastics), which demonstrate the following characteristics: a) directly cytotoxic with no active metabolites; b) neutropenic at the MTD; c) accompanying animal and human haematology data; d) similar, or at least known, protein binding across the species; and e) accompanying animal and human pharmacokinetic data. These comparisons will enable the validity of the *in vitro* assay to be determined, while minimising the possibility that erroneous conclusions are drawn because of the complicating nuances described previously.

In the future, it is envisaged that validated *in vitro* assays could help to solve various problems during the regulatory review of preclinical haematotoxicity data. Since myelotoxicity is the most common dose-limiting toxicity with anticancer drugs, and because *in vitro* assays essentially measure differences in the potencies of compounds between species, comparative *in vitro* studies could be undertaken to identify those compounds which are significantly more toxic to humans than to either dogs or rodents. It is important to be able to identify such compounds, because there is a definite risk of lethal overdose in the first cohort of patients to which they are administered; this risk cannot be identified during preclinical testing.

It should be possible to adjust the starting doses for Phase I trials of antineoplastics based on relative drug sensitivities between species. There is a traditional 10-fold, or greater, safety factor built into calculations of the human starting dose based on the MTD determined during preclinical testing; this accommodates uncertainties regarding species differences in target organ sensitivity, pharmacokinetics, metabolic activation, and plasma protein binding. If it were possible to incorporate in these calculations potency differences between humans and the test species, the starting doses could be considerably closer to the MTD, without compromising safety. Thus, not only would Phase I trials be completed more quickly, but fewer patients would be treated with ineffective doses.

In this respect, the predictivity of the data obtained from animal toxicology studies could be increased by using *in vitro* tests, and the level of uncertainty about human safety could be decreased. The benefits resulting from modification of the starting dose should be evaluated in a retrospective analysis, by using existing data: a so-called clinical trial "re-enactment", in which the Phase I trials of a series of antineoplastics, for example, are conducted again on paper, to determine the effects of incorporating *in vitro* data into the decision-making process.

This type of analysis is currently being undertaken in a collaborative study between scientists at the US Food and Drug Administration and the National Cancer Institute. The preliminary results suggest that: a) starting dose adjustment based on *in vitro* data is most accurate when very precise IC90 values are compared, which is consistent with the idea of dosing to the MTD; and b) dose adjustment based on *in vitro* data is not accurate for those compounds which show non-haematological dose-limiting toxicities in both canine and rodent models. This type of approach has the potential to considerably reduce animal use in toxicology studies on myelosuppressive compounds.

It will also be important to determine, by the retrospective analysis of existing data, whether any degree of "safety" is lost if *in vitro* assays are employed in the early phases of the development of new compounds, in order to identify the species of laboratory animal which has the sensitivity most similar to humans, so that subsequent *in vivo* toxicology studies are only undertaken in this one species. This approach may need to be confined to those compounds which are consistently haematotoxic across all

species. If appropriate, such an approach could refine and reduce animal use to a considerable extent. If *in vitro* tests enable accurate identification of the pharmacodynamic parameters which determine haematotoxicity and the MTD, then *in vitro*-derived human parameters could be used instead of the values currently derived from laboratory animals for pharmacologically guided Phase I clinical trials (94, 101).

In addition, validated *in vitro* assays with human cells could become useful for rapidly evaluating manipulations of haemopoietic grafts and procedures related to gene therapy employing stem cells.

Conclusions and Recommendations

Validated, predictive, *in vitro* haematotoxicity assays, the relevance and limitations of which are understood, have the potential to substantially reduce the number of animals required for haematotoxicity testing, while also improving its scientific basis, and will definitely enable the refinement of those animal procedures which have to be conducted. These assays will play a key role in bridging the gap between preclinical toxicology studies in animal models and clinical investigations. The use of validated *in vitro* haematotoxicity tests (in particular, those employing human cells) will aid considerably the development of new therapeutic agents, and the data from such tests will help in the risk assessments of food additives and other chemicals and products.

General

1. Highest priority should be given to validation of the CFU-GM assay for predicting acute-onset neutropenia. This is justified on the basis of: a) existing evidence of its probable usefulness for regulatory testing purposes (11, 63, 65, 66, 111-116); and b) similarities in the protocols for CFU-GM assays which have been derived independently by several laboratories.

2. ECVAM should initiate and coordinate an inter-laboratory study designed to validate optimised protocols for CFU-GM assays with human, rodent, and canine tissues, and should be involved in providing standardised reagents (cell preparations, sera, tissue culture media, cytokines, etc.), and in the data collection and analysis for the study. The

validation study should determine the reproducibility of the methodology and its ability to provide data predictive for the severity of the neutrophil nadir. Following validation of the methodology, the predictive power of the CFU-GM assay should be evaluated by testing cytotoxic neutropenic compounds, and then undertaking: a) qualitative comparisons of the *in vitro* and *in vivo* data (for this, the test compounds should be selected such that the *in vitro* and *in vivo* schedule dependencies of their toxicities are identical; the *in vitro* and *in vivo* rankings of the sensitivities of the species to them are identical; and there are known differences in their effects on neonatal and adult haemopoietic cells from animal models); and b) quantitative comparisons of the *in vitro* and *in vivo* data (in this respect, the criteria to be met include identical percentage reductions in the progenitor colonies *in vitro* and in the neutrophil counts at nadir *in vivo* at comparable exposure levels to the toxicant; 93).

3. A database should be established which contains existing human and animal *in vivo* data on acute and chronic haematotoxicity, for use in the validation of *in vitro* assays. Existing and future *in vivo* and *in vitro* data should be added to the database in a systematic manner.

4. A retrospective analysis of existing data should be undertaken, to determine whether the use of only one animal species for toxicological studies (on compounds showing dose-limiting haematotoxicity in all of the preclinical test species), to be selected on the basis of comparative *in vitro* haematotoxicity data, would have affected the regulatory decision taken with respect to human safety.

5. Clinical trial "re-enactments" should be conducted, to establish whether adjustment of the starting doses of myelosuppressive anticancer drugs in Phase I clinical trials, based upon comparative *in vitro* haematotoxicity data, would accelerate trial completion without compromising patient safety.

In Vitro Testing

6. Due to its high turnover rate, bone marrow is sensitive to compounds which cause general cytotoxic effects or which are genotoxic, leading not only to cytopenia but also increasing the risk of

developing leukemia. The cytotoxic and genotoxic effects of compounds can be evaluated by other established test methods; primary bone marrow cultures are not considered to be necessary for this purpose.

7. The stability of the test compound *in vitro* should be determined. If it is unstable, the activities of the decomposition products need to be investigated. The rate of decomposition should be measured, in order to calculate the level of toxicant exposure (AUC) and to determine the duration of exposure *in vitro*.

8. If the compound is likely to undergo biotransformation *in vivo,* the putative metabolites, as well as the parent compound, should be tested *in vitro*. The possibility of species-specific biotransformation should be borne in mind, which may necessitate the use of human liver microsomes as a metabolising system, or the need to undertake studies with human hepatocytes or liver slices.

9. The capacity of bone marrow stroma to bioactivate chemicals, resulting in the production of reactive species, should be fully characterised. The relative importance of this metabolic activity for chemical-induced haematotoxicity should be elucidated.

10. There are several sources of human progenitor cells, including the mononuclear fractions from bone marrow, umbilical cord blood and peripheral blood. The $CD34^+$ cells from each of these sources can also be used for *in vitro* studies. Bone marrow-derived or peripheral blood-derived progenitors might be the most appropriate for human toxicology studies, but cord blood-derived cells are more readily available. A study should be conducted to determine the relative sensitivities of human CFU-GM in these two sources to direct-acting haematotoxins with well-characterised haematotoxicity profiles *in vivo*. If they are of equal sensitivity, cord blood cells, or sub-populations of these, should be used for further (validation) studies.

11. Colony-forming assays other than the CFU-GM assay (CFU-G, CFU-GEMM, CFU-E, BFU-E, CFU-MK) should also be standardised and validated. These assays are currently used for studying the proliferative capacities of progenitors from various lineages (11, 12, 14). Their ability to provide data

predictive for *in vivo* cytopenia following single dose exposure should be determined. Priority should be giv n to the validation of: a) CFU-G assays for predicting acute neutropenia with nadir within 6-9 days of exposure in humans and dogs, and within 2-4 days of exposure in rodents; and b) CFU-GEMM assays for predicting delayed neutropenia with nadir within 20-40 days of exposure in humans and dogs, and within 10-20 days of exposure in rodents. In addition, it should be determined whether the inhibition of BFU-E or CFU-E *in vivo* following a single or brief multi-day exposure to the toxicant results in any decrease in peripheral erythrocyte counts.

12. Although colony-forming assays for haemopoietic progenitors are considered useful for predicting acute toxicity, stem cell assays are required for assessing delayed haematotoxic effects resulting from chronic or multiple exposures which perturb bone marrow homeostasis and stimulate stem cell repopulation of the progenitor pool, thereby increasing the sensitivity of normally dormant stem cells to toxicants. For example, this can result from chronic exposure to low doses of environmental toxins and food additives. Consideration should be given to whether *in vitro* stem cell assays should incorporate the quantification of mature blood cell output and/or the production of progenitors as (an) endpoint(s). New screening tests employing stem cells are also required.

13. Attention should be focused on the development of *in vitro* assays for the prediction of thrombocytopenia, which can be a serious side effect of cytoreductive therapy in cancer patients. An appropriate *in vitro* endpoint for megakaryocytopoiesis *in vitro* should be identified, which correlates well with platelet levels *in vivo.*

14. Evidence from *in vivo* and *in vitro* studies indicates that, with some compounds, the bone marrow microenvironment is the major target for haematotoxicity. ECVAM should stimulate investigations which focus on determining which systems could be used to evaluate toxic effects on the haemopoietic microenvironment. Initially, it is suggested that effects on the proliferative capacities of the stromal precursors (the CFU-F assay), and on the ability of the mature microenvironment to support

haemopoiesis, should be studied.

15. Immortalised stromal cell lines, and haemopoietic stem and progenitor cell lines, which mimic the haemopoietic and pharmacological properties of primary stromal, stem, and progenitor cells, respectively, should be established from relevant species (including humans). These should be made available to research scientists through international cell repositories. Such cell lines could replace the use of primary cultures for *in vitro* haematotoxicity testing (in particular, for screening purposes) and reduce the use of animals considerably. In addition, *in vitro* analyses of the complex interactions between stem cells and stromal cells could be standardised more readily by using established cell lines.

16. *In vitro* manipulation of haemopoietic grafts may result in haematotoxic effects. The immediate and long-term effects associated with stem cell labelling with monoclonal antibodies, and with culturing cells in the presence of haemopoietic growth factors and media supplements, should be carefully evaluated. The potential haematotoxic effects resulting from the insertion and expression of foreign genes in primary precursor cells can be investigated *in vitro* by using both short-term and long-term assays. Special attention should be paid to each construction, to ensure that the self-renewal and multi-potential functions of the haemopoietic stem cells are not impaired, at least in the *in vitro* systems.

17. Colony-forming progenitor assays should be able to predict acute cytopenia resulting from a single exposure to a compound. However, to enable the toxic effects of intermittent, multiple and/or prolonged exposures to be predicted, as well as delayed-onset or irreversible haematotoxic effects, new *in vitro* technologies for culturing haemopoietic cells should be developed, which model human *in vivo* haemopoiesis in terms of multi-lineage cell production (myeloid and lymphoid), cell cycle kinetics, exposure of the progenitors to test compound, relevant extracellular levels of human macromolecules and metabolites, and pharmacological characteristics. Assays which model *in vivo* haemopoiesis more completely should be useful for detecting toxic effects on progenitors which are due to non-cytotoxic mechanisms, such as premature differentiation and slower cell cycling.

Use of In Vitro Data for Predicting Haematotoxicity In Vivo

18. Th interpretation of *in vitro* data, and their extrapolation to the *in vivo* situation, should be conducted on a pharmacological basis, taking into consideration the duration of exposure, and the effective concentration and metabolism of the compound.

19. The use of different sera and other protein components in culture media can markedly influence the concentration of the free (unbound) compound, thereby affecting the outcome of the toxicity test. Thus, to facilitate *in vitro/in vivo* extrapolation, the concentration of the free compound should be determined under the particular assay conditions, and the endpoint measured should be expressed with respect to this free concentration.

20. For inter-species and *in vitro/in vivo* comparisons, the IC (inhibitory concentration) values used should be those which are most relevant to the level of haematotoxicity *in vivo* considered to be acceptable for the particular test compound. For example, IC90 values are more appropriate for antineoplastics, whereas IC0 (NOEL) values should be used for food constituents and environmental contaminants.

21. Haematotoxicity *in vivo* is often dependent on the schedule of compound administration; it should always be confirmed that *in vitro* assays reflect this dependency.

22. To improve the predictive values of *in vitro* assays for human haematotoxicity, human haemopoietic tissue, from donors with steady-state haemopoiesis and who are free/not at risk from microbial infection, should be used whenever possible. An exception to this would be when studies are being undertaken with substances which are haematotoxic only in a specific sub-group of the human population.

23. Due to the complexity of the *in vitro/in vivo* relationship, an important next step should be to test a number of cytotoxic compounds (antineoplastics) known to possess straight-forward pharmacological characteristics (for example, a direct mechanism of action, no cytotoxic metabolites, and a high degree of chemical stability *in vitro).* The test compounds

should exhibit dose-limiting neutropenia, for which comprehensive human and animal haematotoxicity data are available.

24. For anticancer drugs, retrospective studies should be undertaken to establish the predictive value of the *in vitro* CFU-GM assay for *in vivo* myelosuppression, which is observed in Phase I clinical trials and in preclinical studies conducted in various animal species.

25. Standardised *in vitro* colony-forming assays for progenitors of different lineages should be evaluated, to assess whether they are able to predict the reduction in the total number of progenitors which occurs *in vivo* (and which results in various degrees of acute cytopenia following exposure to a single dose of the toxicant).

26. It should be determined whether *in vitro* assay systems which include the microenvironment are better than conventional clonogenic assays for predicting haematotoxicity *in vivo.*

27. Age-dependent differences in long-term haemopoietic dysfunctions have been described. Stromal failure is considered to be critical with respect to the generation of these effects. *In vitro* tests with neonatal and adult haemopoietic cells should be compared, to investigate whether the data obtained with various chemicals reflect the known *in vivo* spectrum of sensitivity which is observed in laboratory animals of different developmental ages.

28. In all test species, clinical leucocyte counts should always be accompanied by differentials, so that the *in vitro* data for the myeloid lineage can be related to granulocyte or monocyte counts, rather than to the total leucocyte (WBC) count. Total leucocyte counts are not a useful endpoint, because of major differences between species in the relative contributions of lymphocytes and granulocytes. In rodents, there should be little, if any, relationship between CFU-GM inhibition *in vitro* and leucocytopenia *in vivo*, unless, by chance, lymphopoiesis and myelopoiesis are affected to similar extents.

Future Research Needs

29. Research priorities are: a) the development of assays which can be used for the prediction of chronic, or delayed-onset, myelotoxicity; and b) the identification of *in vitro* endpoints which are relevant for investigating the putative haematotoxic effects of chemicals in a more time-effective and cost-effective manner than the use of clonogenic assays and long-term cultures.

30. New cellular and molecular markers should be identified which may eventually refine or replace the *in vitro* endpoints used at present (such as those in the clonogenic assays). Only *in vitro* endpoints closely related to an *in vivo* haematotoxic effect will be of use in this respect. The importance of membrane markers, cell adhesion molecules, the extracellular matrix, and cytokine expression and production, should be explored further by using flow cytometry and gene expression assays. The identification of surrogate markers should enable the development of tests which are faster to undertake, leading to greater throughput of chemicals. The surrogate markers need to be closely linked statistically to the actual (validated) endpoint (a positive correlation of at least 90%), so that the predictive value of the assay is not compromised.

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Highly Potent RANTES Analogues either Prevent CCR5-Using Human Immunodeficiency Virus Type 1 Infection In Vivo or Rapidly Select for CXCR4-Using Variants†

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The natural ligands for the CCR5 chemokine receptor, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES (regulated on T-cell activation, normal T-cell expressed and secreted), are known to inhibit human immunodeficiency virus (HIV) entry, and N-terminally modified RANTES analogues are more potent than native RANTES in blocking infection. However, potent CCR5 blocking agents may select for HIV-1 variants that use alternative coreceptors at less than fully inhibitory concentrations. In this study, two N-terminal chemical modifications of RANTES produced by total synthesis, aminooxypentane (AOP)-RANTES[2-68] and *N*-nonanoyl (NNY)-RANTES[2-68], were tested for their ability to prevent HIV-1 infection and to select for coreceptor switch variants in the human peripheral blood lymphocyte-SCID mouse model. Mice were infected with a CCR5-using HIV-1 isolate that requires only one or two amino acid substitutions to use CXCR4 as a coreceptor. Even though it achieved lower circulating concentrations than AOP-RANTES (75 to 96 pM as opposed to 460 pM under our experimental conditions), NNY-RANTES was more effective in preventing HIV-1 infection. However, in a subset of treated mice, these levels of NNY-RANTES rapidly selected viruses with mutations in the V3 loop of envelope that altered coreceptor usage. These results reinforce the case for using agents that block all significant HIV-1 coreceptors for effective therapy.

Primate lentiviruses initiate infection by binding to two cell membrane receptors, CD4 (24) and one of several chemokine receptors (1, 4, 6, 7, 10, 17, 18, 20). CCR5 is the coreceptor used by primary, macrophage-tropic human immunodeficiency virus type 1 (HIV-1) isolates which are most frequently transmitted between humans (13, 14). The CXCR4 chemokine receptor is utilized by T-cell-line-adapted HIV-1 isolates (6, 20), and viruses using this coreceptor are isolated from about one-half of infected individuals late in the course of disease (37). Viruses using CCR5 or CXCR4 coreceptors are now termed R5 and X4, respectively (5). Several other chemokine receptors, including CCR2b, CCR3, STRL33, and gpr15 and gpr1 can mediate virus entry (2, 9, 19), but CCR5 appears to be the most widely expressed and utilized (40). The natural ligands for CCR5 are the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated on T-cell activation, normal T-cell expressed and secreted) (12). N-terminal modifications of RANTES result in antagonists that can block HIV-1 infection without signaling calcium flux (23, 32, 35). These modifications include N-terminal truncation (RANTES[9-68]) (3) and the addition of methionine (32) or the substitution of Ser-1 of RANTES by the *n*-pentane oxime of glyoxylic acid (AOP) at the N terminus of RANTES (35). AOP-RANTES was particularly effective at blocking infection with R5 HIV-1 isolates in vitro, perhaps due to its inhibition of receptor recycling (23).

These observations led us to investigate the activity of AOP-RANTES and a novel N-terminal modification, N^{α}-nonanoyl-RANTES[2-68] (hereafter referred to as NNY-RANTES), as antagonists of HIV-1 infection in a small animal model. SCID mice repopulated with human peripheral blood mononuclear cells (hu-PBL-SCID mice) are highly susceptible to HIV-1 infection by a variety of isolates, including R5, R5X4, and X4 viruses with minimal sequence differences (26–28, 31). A major concern about antagonists for a single chemokine coreceptor is their potential to select for viruses that use alternative coreceptors. Although the evolution from R5 to X4 viruses is very slow in patients, the selective pressure of a potent CCR5 blocking agent might rapidly select for X4 variants. To address this concern experimentally, we used virus derived from the 242 molecular clone to infect hu-PBL-SCID mice, since this R5 isolate needs only a single amino acid substitution to become R5X4, and it needs only three changes to become X4 (8, 36).

MATERIALS AND METHODS

Synthesis of AOP- and NNY-RANTES. N-terminal-modified chemokines were prepared by total chemical protein synthesis as previously described (38). The N-terminal modifications were incorporated by an on-resin reaction of RANTES [2-33] with the preformed oxime *n*-pentyl-*O*-N=CHCOOH as the last step in the chain assembly to give AOP-RANTES[2-33] thioester or with nonanoic acid to give N^{α}-nonanoyl-RANTES[2-33] thioester. Native chemical ligation (16) of the purified unprotected peptide segments of AOP-RANTES[2-33] thioester with RANTES[34-68] in aqueous buffer gave the full-length polypeptide produced in reduced form, which was folded with disulfide formation in aqueous buffer and purified by reversed-phase high-pressure liquid chromatography (HPLC). The folded AOP-RANTES was homogeneous on HPLC and gave a molecular mass of 7,901.02 ± 0.8 Da on electrospray ionization mass spectroscopy (calculated

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average isotope composition, 7,901.2 Da). NNY-RANTES was similarly prepared by chemical ligation of N^{α}-nonanoyl-RANTES[2-33] thioester with RANTES[34-68]. The folded NNY-RANTES was homogeneous on HPLC and gave a molecular mass of 7,899.96 ± 0.01 Da on electrospray ionization mass spectroscopy (calculated average isotope composition, 7,900.21 Da). Large amounts (>50 mg) of purified folded proteins were obtained from a single research scale synthesis of each analogue. Multidimensional nuclear magnetic resonance measurements showed that both N-terminal analogue proteins were conformationally homogeneous (data not shown).

Generation of hu-PBL-SCID mice. C.B-17 SCID mice were bred under specific-pathogen-free conditions at The Scripps Institute and tested for mouse immunoglobulin M (IgM) production at 8 weeks of age. Mice with <5 μg of IgM per ml were engrafted with peripheral blood mononuclear cells (PBMC) prepared from Epstein-Barr virus (EBV)-seronegative donors from the Scripps General Clinical Research Center pool. SCID mice were injected with 20×10^6 PBMC intraperitoneally and checked for plasma levels of human IgG after 12 to 13 days. Mice with >100 μg of human IgG per ml were used for HIV-1 infection. Each experiment used mice generated from a single, different EBV-negative donor. All three donors were genotyped for the CCR5 Δ32 mutation and were homozygous wild type.

HIV-1 virus pools. Infectious stocks of the 242 molecular clone were made by transfecting 293 cells with a full-length molecular clone provided by Bruce Chesebro. Virus recovered from the culture after 48 h was used to infect PBMC cultured for 4 days with phytohemagglutinin (PHA; 2 μg/ml) and for 2 days with interleukin-2 (IL-2; 20 U/ml). Infectious virus was recovered after 7 to 10 days of culture, and the tissue culture infectious dose (TCID) of the virus was determined by endpoint titration. Mice were infected with 1,000 TCIDs of virus. Sequencing results showed that our original 242 infectious stock differed from the published sequence by having an H rather than R in position 21 of the V3 loop (see Table 2). This 242H variant was used for all of the experiments depicted in Fig. 2. A second lot of the 242 clone was prepared subsequently and shown to retain the original sequence. The original sequence was also recovered from one animal (NNY-2 R3 in Fig. 2B), which could have resulted from either mutation or selection of the original R sequence from a virus pool dominated by the 242H variant.

In vitro assays. PBMC were collected from normal blood samples by density centrifugation. $CD4^+$ T cells were separated by depletion of other cell types by antibody treatment and immunomagnetic bead separation. Whole PBMC or separated $CD4^+$ T cells were cultured at 5×10^4 cells per well in 96-well microtiter plates. Cells were activated with PHA and IL-2 for 3 to 4 days, the medium was replaced with concentrations of AOP- or NNY-RANTES ranging from 12,660 to 1.27 pM (100 ng/ml to 1 pg/ml), and the cells were incubated for 30 min at 37°C and then infected with 100 TCIDs of HIV-1 in the continued presence of modified RANTES. After overnight incubation, free virus was removed, and fresh medium containing the original concentration of modified RANTES was added. The wells were sampled on days 4, 7, and 10 after infection, and p24 HIV capsid antigen was measured by enzyme-linked immunosorbent assay (ELISA) (NEN Life Sciences, Boston, Mass.).

Administration of CCR5 antagonists to mice. AOP- or NNY-RANTES were dissolved in 0.9% saline at 2.5 mg/ml (316 μM). Alzet 2001 mini-osmotic pumps (ALZA Pharmaceuticals, Palo Alto, Calif.) were loaded with 200 to 225 μl of compounds or bovine serum albumin (BSA) as a control. Pumps were surgically implanted subcutaneously under halothane anesthesia between the scapulae, and the incision was closed with a single wound clip. Pumps were observed for proper placement during the course of the experiment. A single intraperitoneal (i.p.) injection of 1 mg in 0.4 ml of either RANTES compounds (126.6 μM) or BSA was administered just prior to virus infection. These concentrations were based on the solubility and the availability of the compounds and not on prior pharmacokinetic studies.

Virus infection in mice. Infection of hu-PBL-SCID mice with HIV-1 was determined by plasma HIV-1 RNA levels measured by the quantitative Roche PCR assay (Amplicor HIV Monitor; Roche Molecular Systems, Somerville, N.J.). The limit of detection was 200 to 400 copies/ml, depending on the plasma volume available. Depletion of $CD4^+$ T cells was measured by flow cytometry. Cells recovered from the peritoneal cavity or regional lymph nodes of hu-PBL-SCID mice were stained with fluorescein- or phycoerythrin-labeled antibodies to human CD3, CD4, CD8, or CD45 and mouse H-$2K^d$ (Pharmingen, San Diego, Calif.) and analyzed with a FACScan (Becton Dickinson, Mountain View, Calif.) flow cytometer. $CD4^+$ T cells are expressed as a percentage of total $CD3^+$ cells recovered.

RANTES levels in mice. Plasma from hu-PBL-SCID mice was analyzed for RANTES antagonists by ELISA (R & D Systems, Minneapolis, Minn.) by using standard curves for either AOP- or NNY-RANTES. Plasma was diluted either 1:10 or 1:100 to bring the RANTES concentration into the optimal sensitivity range of the assay.

V3 envelope sequences. RNA was extracted from mouse plasma by using the Qiagen viral RNA kit (Qiagen, Valencia, Calif.). RNA was converted to cDNA by reverse transcriptase PCR. cDNA was amplified by nested PCR with the following primers: outer V3 sense, CCAATTCCCATACATTATTG; outer V3 antisense, ATTACAGTAGAAAAATTCCCC; inner V3 sense, CAGTACAATGT ACACATGGAATT; and inner V3 antisense, AATTTCTGGGTCCCCTCCT GA. The final 356-bp product was cloned by using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif.), and the resulting product was subjected to automated sequencing (ABI; Perkin-Elmer, Foster City, Calif.). The final sequence encodes 54 amino acids 5′ of V3 and 50 amino acids 3′ of V3. Although only the translated V3 sequence is reported in Table 2, the entire sequence was examined, and there were no mutations outside of V3. Note that HIV-1 242 has a V3 region of only 34 amino acids compared to the clade B consensus length of 35. The consensus G at position 24 is deleted in the 242 clone (8), so substitutions 3′ to this deletion are aligned to the consensus sequence (i.e., no position 24 residue exists in these sequences).

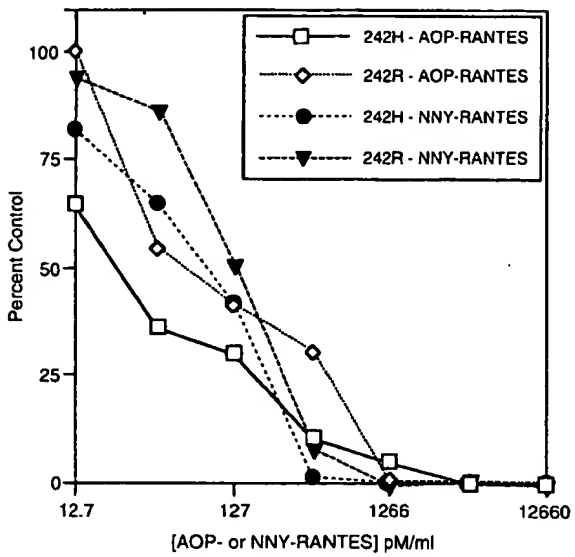

FIG. 1. Inhibition of HIV-1 infection by AOP- and NNY-RANTES in cultured primary human PBMC. Virus replication was measured by p24 capsid antigen production after 5 to 7 days of infection. Infection was with two R5 variants of HIV-1 242: the original 242 isolate with an R at position 21 of V3 and a spontaneous mutant with H at position 21. These viruses are referred to as 242R and 242H, respectively.

Use of coreceptors. The coreceptor usage of viruses recovered in these experiments was tested by two independent methods. First, the viruses were used to infect PHA- and IL-2-activated PBMC cultures derived from a donor who is homozygous for the CCR5 Δ32 mutation (22) and thus fails to express CCR5. Second, virus isolates were grown on GHOST cells transfected with either CXCR4 or CCR5 and a reporter construct encoding enhanced green fluorescent protein. These cell lines were obtained through the NIAID/NIH AIDS Research and Reference Reagent Program and were contributed by Vineet N. KewalRamani and Dan R. Littman. Infection of GHOST cell lines was detected by flow cytometry at 5 days after the addition of virus.

RESULTS

Inhibitory activity of AOP- and NNY-RANTES in vitro. The ability of AOP-RANTES and NNY-RANTES to inhibit R5 virus infection, including the R5 242 isolate of Chesebro et al. (8), was confirmed by in vitro experiments. The results show that both AOP-RANTES and NNY-RANTES were effective at inhibiting the infection of activated PBMC with the R5 SF162 isolate (data not shown) as well as with the two variants of the R5 242 HIV-1 isolate (Fig. 1, also see Table 2). Both AOP-RANTES and NNY-RANTES failed to inhibit infection with X4 isolates (data not shown). In contrast to the more potent inhibition of SF162 (26a), ADA, and JR-CSF (30a), R5 HIV-1 isolates, NNY-RANTES was not more potent than AOP-RANTES at inhibiting the replication of either the 242H or 242R variants in vitro. HIV-1 242R (with an R rather than an H at position 21 of V3) was more resistant to inhibition than was 242H with either of the two CCR5 antagonists. This suggests that minor sequence changes in V3 may impact the affinity of the envelope-CCR5 interaction. AOP-RANTES thus demonstrates the previously observed specificity for CCR5-

using HIV-1 isolates (35), and NNY-RANTES has a similar specificity and equal or higher potency (30a). The 242H isolate was used for all subsequent experiments.

Activity of AOP- and NNY-RANTES in hu-PBL-SCID mice. We performed three replicate experiments in hu-PBL-SCID mice to evaluate the in vivo efficacy of AOP- or NNY-RANTES. Because we anticipated rapid clearance from plasma and wished to maintain stable levels of the CCR5 antagonists, they were administered at the rate of 316.5 nM (2.5 μg)/h by continuous infusion by using subcutaneously implanted osmotic pumps. In addition, a single dose of 126.6 μM (1 mg; ~50 mg/kg) of each antagonist was injected i.p. just prior to virus infection. Serial plasma HIV RNA determinations were performed on the treated and control hu-PBL-SCID mice after infection with HIV-1 242. In the experiment shown in Fig. 2A, mice were infused with AOP-RANTES or BSA as a control. Plasma concentrations of AOP-RANTES ranged from 157 to 604 pM on day 7 of infusion (Table 1, experiment A). Two of the four mice treated with AOP-RANTES had undetectable viral RNA levels at the end of the 7-day infusion period, but virus levels increased in all mice once AOP-RANTES administration was halted. Thus, as used here, AOP-RANTES was capable of reducing viral load, but it could not prevent HIV-1 infection despite plasma levels that were fully inhibitory in vitro (Fig. 1).

We therefore tested the inhibitory capacity of NNY-RANTES in the next two experiments. Infusion of NNY-RANTES followed the same dose and schedule as AOP-RANTES (126.6 μM or a 1-mg bolus injection given i.p. followed by 316.5 nM or 2.5 μg/h delivered with an Alzet pump) but led to a mean plasma concentration of 96 pM (Table 1, experiment B) on day 7 of infusion, a level with less than complete inhibitory activity in vitro (Fig. 1). Nonetheless, four of five hu-PBL-SCID mice infused with NNY-RANTES had undetectable viral RNA levels on day 7 of the infusion period, and only one additional animal subsequently developed viremia (NNY-2 R3 in Fig. 2B). NNY-RANTES treatment was thus successful in preventing R5 HIV-1 infection in three of five mice, despite achieving five- to sixfold lower plasma concentrations than AOP-RANTES. This experiment was repeated with a different human donor to generate hu-PBL-SCID mice to confirm inhibition of infection at such low concentrations of NNY-RANTES. This experiment also used the same dose and schedule of NNY-RANTES administration and resulted in even lower mean plasma concentrations of NNY-RANTES (75 pM in Table 1, experiment C). However, the inhibition of virus infection was similar to the previous experiment, with NNY-RANTES preventing infection in three of five mice (Fig. 2C). Virus and viral sequences from the two mice that became infected were further characterized (see Table 2, below). NNY-RANTES thus was able to prevent HIV-1 infection in 6 of 10 hu-PBL-SCID mice (Fig. 2B and C) at plasma concentrations lower than the 50% inhibitory dose for HIV-1 242H (~150 pM; Fig. 1) in vitro.

We also measured the relative survival of human $CD4^+$ T lymphocytes in hu-PBL-SCID mice treated with each CCR5 antagonist. Table 1 compares the recovery of $CD4^+$ T cells (as a percentage of the total $CD3^+$ T cells) in the peritoneal cavity of individual mice treated either with BSA or with AOP- or NNY-RANTES at 2 weeks after infection. Both AOP- and NNY-RANTES were able to slow the depletion of $CD4^+$ T cells, even in mice where HIV-1 infection was not prevented.

NNY-RANTES but not AOP-RANTES selected for coreceptor switch variants under these experimental conditions. To determine whether virus from hu-PBL-SCID mice that became infected despite treatment with AOP- or NNY-RANTES was evading the antagonists by mutating from CCR5 to CXCR4

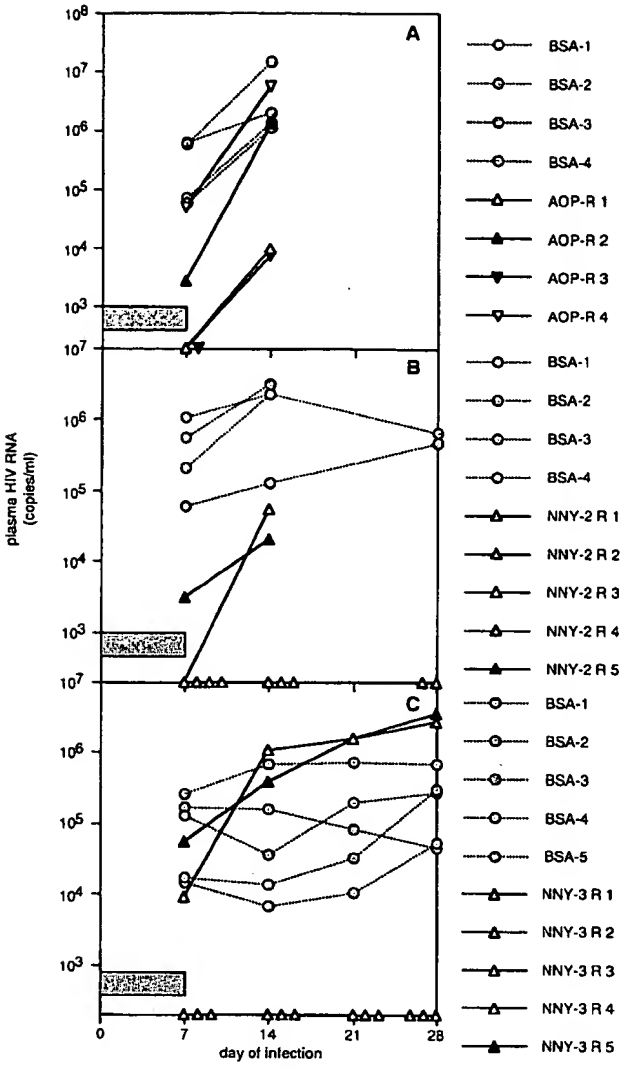

FIG. 2. Inhibition in hu-PBL-SCID mice of HIV-1 infection by AOP- or NNY-RANTES. CCR5 antagonists were delivered by subcutaneously implanted osmotic pumps at the rate of 316 nM (2.5 μg)/h beginning 1 day before infection with the 242H isolate (see the text). A single dose of 126.6 μM (1 mg) of AOP-RANTES (A) or NNY-RANTES (B and C) was administered just prior to HIV-1 challenge. All animals thus received a bolus injection of compounds just prior to infection and continuous infusion of compounds from day −1 to at least day 7 after infection, as indicated by the horizontal bar in each panel. Data presented are plasma HIV RNA copies per milliliter at 1 to 4 weeks after infection, and each point represents the value for a single animal at each time point. Data collection was halted after 2 weeks in the first experiment (panel A), since all mice were positive. Two mice from each treatment group were sampled for human cell survival after 2 weeks of infection in the second experiment (panel B), so fewer values are recorded at later time points.

coreceptor utilization, we amplified proviral DNA envelope genes directly from hu-PBL-SCID mouse tissue and sequenced the region surrounding the V3 loop, a critical determinant of coreceptor usage (11). V3 sequences observed in the mice are shown in Table 2. In the first experiment (Fig. 2A), sequences obtained after 2 weeks of infection from all mice treated with AOP-RANTES were identical to the starting 242 virus isolate (which was found to contain an H in place of the published R at position 21, a change that had occurred prior to the initiation of these experiments). In the second experiment (Fig. 2B), HIV-1 sequences recovered after 4 weeks of infection from the

TABLE 1. Plasma levels of AOP- or NNY-RANTES after 7 days of constant infusion, recovery of $CD4^+$ human T cells, and plasma levels of HIV RNA in hu-PBL-SCID mice treated with CCR5 antagonists

Expt	Mouse[a]	Day 7 plasma AOP- or NNY-RANTES concn (pM/ml)	Day 14 % $CD4^+$ T cells[b] in peritoneal cavity	Day 14 plasma level of HIV RNA (log_{10} copies/ml)
A	AOP-R 1	571	17.1	3.98
	AOP-R 2	604	29.2	6.07
	AOP-R 3	510	27.7	3.86
	AOP-R 4	157	14.3	6.72
	Mean ± SE	460 ± 51	22.1 ± 3.7	5.16 ± 0.73
	BSA-1	<0.01	3.5	7.16
	BSA-2	<0.01	11.9	6.06
	BSA-3	<0.01	6.2	6.31
	BSA-4	<0.01	4.4	6.12
	Mean ± SE		n6.5 ± 1.9	6.41 ± 0.25
B	NNY 2 R1	104	86.1	<2.30[c]
	NNY-2 R2	86		<2.30
	NNY-2 R3	110	32.7	4.72
	NNY-2 R4	87		<2.30
	NNY-2 R5	90	47.9	4.32
	Mean ± SE	96 ± 5	55.6 ± 15.9	
	BSA-1	<0.01	11.7	6.49
	BSA-2	<0.01	12.3	6.36
	BSA-3	<0.01		5.12
	BSA-4	<0.01		6.34
	Mean ± SE		12.0 ± 0.3	6.08 ± 0.32
C	NNY-3 R1	62	ND	<2.30
	NNY-3 R2	52	ND	<2.30
	NNY-3 R3	156	ND	<2.30
	NNY-3 R4	48	ND	6.02
	NNY-3 R5	54	ND	5.59
	Mean ± SE	75 ± 20		
	BSA-1	<0.01	ND	4.16
	BSA-2	<0.01	ND	3.83
	BSA-3	<0.01	ND	5.85
	BSA-4	<0.01	ND	4.56
	BSA-5	<0.01	ND	5.21
	Mean ± SE			4.72 ± 0.36

[a] Each mouse is individually designated as in Fig. 2. AOP-R or NNY-R means the animal was treated with either AOP- or NNY-RANTES.
[b] $CD4^+$ T cells are reported as a percentage of total CD3 T cells recovered from the peritoneal cavity of each animal. ND, not determined.
[c] Below the limit of detection of 200 copies/ml.

two mice that became infected despite treatment with NNY-RANTES differed. One mouse had the sequence of the starting 242 isolate (except for one clone with a replacement mutation at position 30), while the other mouse showed a reversion of the H at position 21 to the R present in the original molecular clone. The presence of H or R at position 21 in these isolates did not impact CCR5 usage but may have impacted susceptibility to NNY-RANTES (Fig. 1) and the rate of replication in vitro (Fig. 3B). These results show that although sequence variation was occurring in this experiment and there may have been selection for sequence variants (either preexisting or generated by mutation) that were less sensitive to NNY-RANTES inhibition, there was not rapid selection for HIV-1 variants that used alternative coreceptors for viral entry. However, viral sequences amplified after 4 weeks of infection directly from two hu-PBL-SCID mice that became infected despite NNY-RANTES treatment (mice NNY-3 R4 and NNY-3 R5) in the third experiment (Fig. 2C) revealed the same three replacement mutations in V3 (Table 2, part C), although there were no other replacement or silent mutations in the remainder of the 356-bp PCR product (data not shown). The changes of S to R at position 11, H to R at position 21, and E to K at position 25 conferred upon these viruses reduced susceptibility to NNY-RANTES and the ability to use CXCR4 for infection. These two viral variants showed reduced sensitivity to inhibition by NNY-RANTES in primary PBMC cultures (Fig. 3A). The ability to use CXCR4 for virus entry was demonstrated by infection of CD4-transfected HeLa cells (MAGI cells [34], which are not permissive for R5 viruses [data not shown]) and by infection of PBMC cultures from an individual homozygous for the CCR5 Δ32 mutation (Fig. 3B). The coreceptor usage of the isolate from mouse NNY-3 R4 was further confirmed by the infection of GHOST cells transfected with either CXCR4 or CCR5. The results are shown in Fig. 4. The 242H isolate used for infection could infect only cells expressing CCR5 (Fig. 4A and B), but the NNY-3 R4 isolate with mutations in the V3 region was capable of infecting both CXCR4- and CCR5-expressing target cells (Fig. 4C and D). The V3 sequences present in the NNY-RANTES-treated animals showed rapid reversion to the 242H parental sequence during in vitro culture (a mixture of variant, parental, and partial revertant sequences were obtained after 7 days of culture [data not shown]), so the properties of the viruses recovered by in vitro propagation reflects a mixture of viral genotypes and phenotypes. This may explain the intermediate levels of sensitivity to NNY-RANTES demonstrated in Fig. 3A. Viruses recovered from tissue cultures containing 12,660 pM (100

TABLE 2. V3 envelope sequences of HIV-1 242 recovered from hu-PBL-SCID mice treated with AOP- or NNY-RANTES

Virus clone[a]	Sequence (1 5 10 15 20 25 30 35)
Expt A	
Stock	CTRPNNNTRRSISIGPGRAFHTT-EIIGDIRQAHC
All clones	----------
Expt B	CTRPNNNTRRSISIGPGRAFRTT-EIIGDIRQAHC
NNY 2 R3-1	----------R----------
NNY 2 R3-2	----------R----------
NNY 2 R3-3	----------R----------
NNY 2 R3-4	----------R----------
NNY 2 R3-5	----------R----------
	CTRPNNNTRRSISIGPGRAFHTT-EIIGDIRQAHC
NNY 2 R5-9	----------
NNY 2 R5-8	----------
NNY 2 R5-4	----------
NNY 2 R5-2	----------
NNY 2 R5-10	----------T-----
Expt C	CTRPNNNTRRSISIGPGRAFHTT-EIIGDIRQAHC
NNY 3 R4-5	----------R---------R---K----------
NNY 3 R4-7	----------R---------R---K----------
	CTRPNNNTRRSISIGPGRAFHTT-EIIGDIRQAHC
NNY 3 R5-1	----------R---------R---K----------
NNY 3 R5-3	----------R---------R---K----------
NNY 3 R5-4	----------R---------R---K----------
NNY 3 R5-10	----------R---------R---K----------
NNY 3 R5-2	----------R---R-----R---K----------

[a] Sequences in part B were derived from the two mice in Fig. 2B. Sequences in part C were derived from the two mice in Fig. 2C. Viruses recovered from mice treated with BSA were also sequenced, and all contained the 242H sequence as in part A.

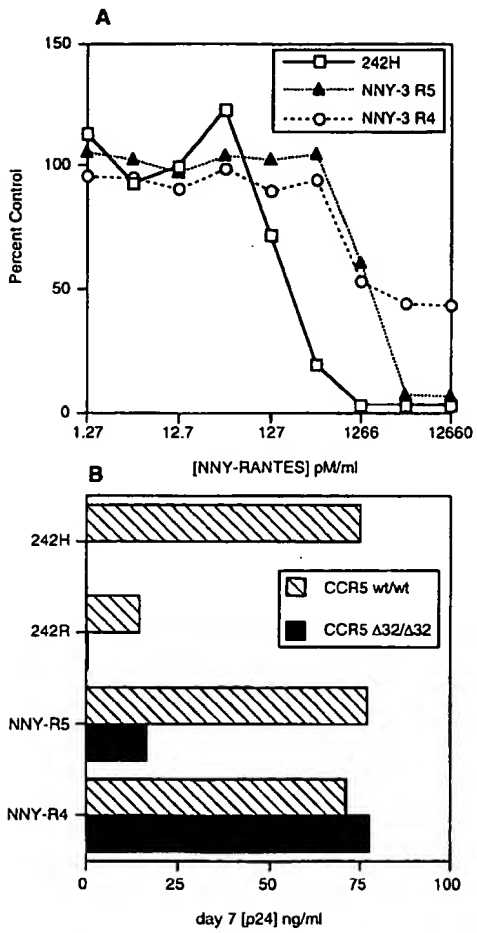

FIG. 3. (A) Inhibition of HIV-1 replication by NNY-RANTES in cultured PBMC. The HIV-1 isolates were the starting 242H virus and the HIV recovered from animals NNY-3 R4 and NNY-3 R5 from the experiment shown in Fig. 2C. These viruses were expanded in vitro for 7 days prior to the addition of NNY-RANTES. Envelope sequences were obtained from the virus at this time point and after 5 days of culture in the presence of 12.7 nM (100 mg/ml) NNY-RANTES. Sequences from the isolate NNY-3 R4 obtained after culture with NNY-RANTES matched the sequence shown in Table 2, part C, obtained directly from the infected hu-PBL-SCID mouse, but sequences obtained before the addition of NNY-RANTES showed several clones with a reversion to a 242 sequence. The NNY-RANTES inhibition data therefore reflect a population of virus sequences, with only a fractional representation of the NNY-RANTES resistant variants. (B) Replication of HIV-1 in cultured PBMC from a normal donor (CCR5 wt/wt) or a donor without CCR5 expression (CCR5 Δ32/Δ32). Both of the starting 242 virus isolates (H or R at position 21 of V3) were unable to replicate in the CCR5-negative cells, but the viruses recovered from mice NNY-3 R4 and NNY-3 R5 were able to replicate in these cells. As noted above, this experiment was done after 7 days of culture in normal PBMC, and there was no longer sequence homogeneity in the NNY-R4 and NNY-R5 isolates at this time.

ng/ml) NNY-RANTES retained the predominant sequence shown in Table 2, experiment C, as did viruses propagated on CCR5-negative cells (as in Fig. 3B), so continued selective pressure was required to maintain this genotype, and such viruses were highly resistant to NNY-RANTES. These results demonstrate that the viral sequences recovered directly from infected cells from mice NNY-3 R4 and NNY-3 R5 represent NNY-RANTES-resistant mutations.

DISCUSSION

These results show that it is possible to block HIV-1 infection with N-terminally modified RANTES compounds in vivo but that the more effective inhibitor was able to select for coreceptor switch variants during the 1-week treatment period (Fig. 2 and 3). Inhibition of virus infection occurred with plasma levels of 50 to 113 pM NNY-RANTES and 500 to 630 pM AOP-RANTES during continuous administration of the antagonists, levels that are lower than the average concentration (~2.5 nM) of native RANTES in human plasma (39) and, for NNY-RANTES, levels that were lower than the in vitro 50% inhibitory concentration (Fig. 1) for the 242H virus isolate. There has been one previous report of a chemokine receptor antagonist (AMD3100) that displayed efficacy against X4 HIV-1 infection in SCID-hu mice, albeit at concentrations of greater than 100 nM (15), but ours is the first report of antiviral activity of a CCR5 antagonist in vivo. The finding that AOP-RANTES was poorly effective at preventing infection and that even NNY-RANTES was not completely effective suggests that the pharmacokinetics of these molecules will need to be manipulated to ensure higher circulating concentrations and that further improvements may have to be made in receptor affinity. It should be noted that even the low doses of NNY-RANTES achieved were similar in activity to a potent neutralizing antibody for the prevention of HIV-1 infection of hu-PBL-SCID mice (21, 30). Mice that were not protected from infection had lower viral RNA levels and higher $CD4^+$ T-cell counts than the controls, suggesting that CCR5 antagonists may be useful in treating established infection.

The duration of the cellular response to CCR5 antagonists is currently unknown. If compounds such as AOP- and NNY-RANTES inhibit virus entry by sequestering CCR5 in the cytoplasm (23), then CCR5 antagonists may have an extended period of activity despite their short half-life in plasma. Alternatively, if antagonists only interfere with gp120 binding by receptor occupancy, then they may need to be constantly present at effective inhibitory concentrations. We chose to administer AOP- and NNY-RANTES by both bolus injection and continuous infusion. It is not clear whether both are required for the observed inhibition of virus infection, but preliminary results suggest that neither a single bolus injection nor continuous infusion alone were sufficient to prevent HIV-1 infection. The steady-state concentrations of NNY-RANTES were lower than those of AOP-RANTES under identical administration conditions. This implies a more rapid turnover of NNY-RANTES, but differential rates of receptor recycling might also have the same effect. The low levels achieved make it even

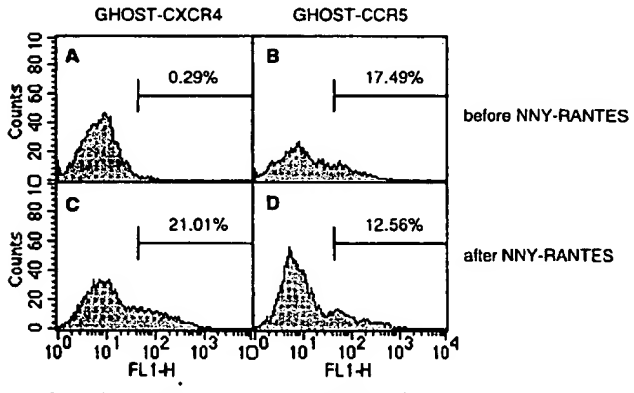

FIG. 4. Coreceptor usage after NNY-RANTES treatment in vivo. The starting 242H isolate and the NNY-3 R4 isolate recovered from an NNY-RANTES treated mouse (Fig. 2C and Fig. 3) were tested for infection of GHOST cells transfected either with CXCR4 (A and C) or CCR5 (B and D). The 242H isolate could only infect GHOST cells expressing CCR5 (B), but the NNY-3 R4 isolate could infect both CXCR4 (C)- and CCR5 (D)-expressing cells.

more surprising that 60% of the challenged hu-PBL-SCID mice were protected from HIV-1 infection by NNY-RANTES.

The emergence of viruses capable of using CXCR4 under the selective pressure of the concentrations of NNY-RANTES used in these experiments demonstrates that the inappropriate use of CCR5 antagonists could generate more pathogenic variants, since there is general agreement that the course of disease progression is accelerated with the switch from R5 to R5X4 viruses (14, 33). The experiments described here were conceived to test this possibility, and the choice of the HIV-1 242 isolate as the challenge virus should have increased the probability of coreceptor switch variants, since it is known that only a single amino change will generate the 241 sequence which is an R5X4 virus (36). However, both of the hu-PBL-SCID mice that developed NNY-RANTES resistant viruses independently generated the same three replacement mutations rather than the E-to-Q substitution at position 24 that distinguishes HIV-1 241 from HIV-1 242 (8). If primary patient R5 isolates require more mutations to generate altered coreceptor usage they may take longer to emerge, but our results suggest that this is very likely to happen. The selective amino acid replacements at positions 11 and 25 of V3, positions known to influence coreceptor usage (8, 25, 29), and the absence of other mutations (either silent or replacement) argue for highly selective pressure exerted by NNY-RANTES treatment under the conditions of these experiments. The H-to-R change at position 21 also occurred in these viruses as well as in the viral variant recovered from one additional mouse (NNY-R3 in experiment 2 [Fig. 2]) and was shown to decrease susceptibility to NNY-RANTES (Fig. 1) as well as the in vitro replication rate (Fig. 3). The rapid reversion of these viruses to the starting sequence in vitro in the absence but not in the presence of NNY-RANTES suggests that the viral variants are less fit for in vitro replication, but they persisted for 3 weeks after cessation of NNY-RANTES treatment in hu-PBL-SCID mice. It is thus not clear that these coreceptor switch variants would be more highly pathogenic than the starting virus in infected humans, although that possibility clearly exists. The rapid selection of CCR5 antagonist-resistant virus mutations observed in hu-PBL-SCID mice suggests that similar experiments could rapidly map the amino acid substitutions required for resistance to these and other antagonists in patient isolates.

These results strongly reinforce the view that clinical use of blocking agents for CCR5 alone would be unwise and that cocktails of antagonists directed toward known coreceptors or antagonists with broader specificity will be required for safe and effective therapy of HIV-1 infection in humans. Nonetheless, the potent activity of NNY-RANTES in preventing infection of 60% of challenged animals at very low concentrations suggests that HIV coreceptors are important targets for current and novel inhibitory agents.

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UNIVERSITY OF NORTH CAROLINA

Full Article

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Macrophage inflammatory protein-1α (MIP-1α) expression plasmid enhances DNA vaccine-induced immune response against HIV-1

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CD8$^+$ cell-secreted CC-chemokines, MIP-1α, and MIP-β have recently been identified as factors which suppress HIV. In this study we co-inoculated MIP-1α expression plasmid with a DNA vaccine constructed from HIV-1 pCMV160IIIB and pcREV, and evaluated the effect of the adjuvant on HIV-specific immune responses following intramuscular and intranasal immunization. The levels of both cytotoxic T lymphocyte (CTL) activity and DTH showed that HIV-specific cell-mediated immunity (CMI) was significantly enhanced by co-inoculation of the MIP-1α expression plasmid with the DNA vaccine compared with inoculation of the DNA vaccine alone. The HIV-specific serum IgG1/IgG2a ratio was significantly lowered when the plasmid was co-inoculated in both intramuscular and intranasal routes, suggesting a strong elicitation of the T helper (Th) 1-type response. When the MIP-1α expression plasmid was inoculated intramuscularly with the DNA vaccine, an infiltration of mononuclear cells was observed at the injection site. After intranasal administration, the level of mucosal secretory IgA antibody was markedly enhanced. These findings demonstrate that MIP-1α expression plasmid inoculated together with DNA vaccine acts as a strong adjuvant for eliciting Th1-derived immunity.

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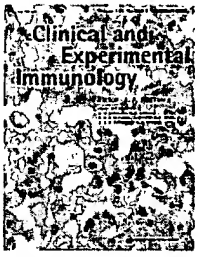
INTRODUCTION

DNA vaccines against HIV-1 have been proven to be an effective means of generating immune responses and protection in a wide variety of preclinical models [1-3]. DNA vaccines provide a means to generate antibodies and cytotoxic T lymphocytes (CTL); they can be a tool for studying mechanisms of antigen presentation, the role of cytokines, and the effects of bacterial DNA in the generation of immune responses; and they also provide a technology for the discovery of novel vaccine antigens. In a previous study, an HIV DNA vaccine consisting of a mixture of cytomegalovirus (CMV) promoter DNA linked to the HIV *env* gene and CMV promoter DNA linked to the *rev* gene (IIIB/REV) induced a certain level of HIV-1-specific humoral and cellular immune responses [4]. However, the immunogenicity of the DNA vaccine was not as strong as expected. The use of expression plasmids as adjuvants for DNA vaccination against AIDS has also been explored to optimize the preparations employed in immunization [5, 6]. DNA co-inoculation can lead to the expression of proteins which may help in inducing a stronger and longer lasting immunity [7-10]. To achieve protective immunity against HIV-1 infection, virus-specific CTL have been shown to play an important role in the clearance of persistent virus infections in both human and animal models [11, 12].



To enhance the HIV-specific cell-mediated immunity (CMI), we tested co-inoculation of the DNA vaccine with MIP-1α expression plasmid. MIP-1α, a member of the β-chemokine family, acts as a chemoattractant for inflammatory cells and modulates functions of monocytes and B and T lymphocytes [13-16], and it also affects haematopoietic stem/progenitor cell growth [17, 18]. Several studies have shown that MIP-1α stimulation enhances interferon-gamma (IFN-γ) production [19], which is essential for the induction of Th1-derived CMI. These observations suggest that MIP-1α would be useful as an effective adjuvant in DNA vaccination by activating macrophages and Th1-type cells. Since DNA is amenable to genetic manipulation, we designed a MIP-1α expression plasmid which we co-inoculated with an immunogenic HIV DNA vaccine [4] to determine whether this plasmid enhances HIV-1-specific immunity.

MATERIALS AND METHODS

Go to: Choose Go

Animals

We used only 6-10-week-old BALB/c female mice purchased from Japan SLC, Inc. (Shizuoka, Japan).

Plasmids

pCMV160IIIB encoding gp160 of HIV-1_{IIIB} and pcREV encoding rev were described previously [4]. Murine MIP-1α cDNA [20] was kindly donated by Dr T. Yoshimura (Department of Immunopathology Section and Laboratory of Immunology, NCI-FCRDC, Frederick). The pCAGGS expression vector [21] was donated by Dr J. Miyazaki (Department of Nutrition and Physiological Chemistry, Osaka Medical University, Osaka, Japan). Murine MIP-1α cDNA was inserted into the Xho I site of the pCAGGS expression vector to obtain the pCAGGSMIP-1α plasmid (Fig. 1).

DNA inoculation

Mice were inoculated by injection or intranasally. A total of 100 μl of DNA mixture containing 2 μg each of pCMV160IIIB and pcREV (hereafter referred to as pCMV160IIIB/REV) and a 5-50 μg dose of pCAGGSMIP-1α diluted in sterile PBS was injected into the right biceps femoral muscle of mice [4]. For the intranasal route, mice were anaesthetized with diethyl ether. After about 20 s, 30 μl of the DNA vaccine preparation containing 2 μg each of pCMV160IIIB/REV and a 1, 10, or 50-μg dose of pCAGGSMIP-1α diluted in PBS were dropped into the nostrils little by little, so as to avoid suffocation [22].

DTH response

Two weeks after DNA inoculation, a total of 25 μl PBS containing 4 μg of the HIV-1_{IIIB} V3 peptide RIQRGPRAFVTIGK was injected into the rear footpads of each mouse. After 24 h, the extent of footpad swelling was measured with a microdial meter (Ozaki Seisakusho, Tokyo, Japan) in units of 10^{-2} mm. Control mice were injected with the same dose of the sperm whale myoglobin peptide ALVEADVA [4, 22].

HIV-1-specific cytotoxic test

As described previously [4], 3 weeks after DNA injection, splenic mononuclear cells were collected and 1 × 10^6 lymphoid cells were restimulated *in vitro* in the presence of the same amount of irradiated (30 Gy) syngeneic spleen cells with 3 μg/ml of the HIV-1 V3 peptide RGPGRAFVTI, a known CTL epitope of HIV-1_{IIIB}. After being cultured for 5 days, the cytotoxic activity of these spleen cells was measured by a 6-h ^{51}Cr-release assay using V3 peptide-pulsed target cells. The target cells were prepared using the same HIV-1 V3 peptide-pulsed P815 cells (H-

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Image Previews

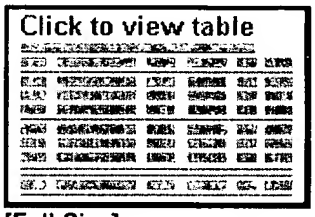

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Table 1. Titres of HIV-1-specific antibody induced by intramuscular (i.m.) and intranasal (i.n.) adm...

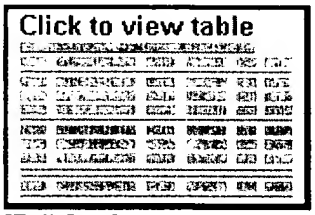

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Table 2. Footpad swelling responses of mice co-inoculated with DNA vaccine and MIP-1α expression plas...

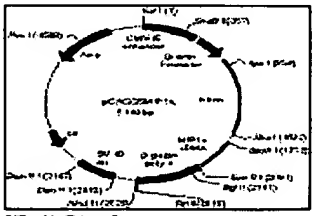

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Fig. 1. Construction of expression plasmid

2^d). The bulk splenocytes used as effector cells were co-cultivated with the target cells at effector-to-target cell (E:T) ratios that ranged from 5:1 to 80:1. Target cell lysis was measured by gamma-ray counting of 100 μl of cell-free supernatant to determine the amount of ^{51}Cr released. The percentage of specific ^{51}Cr released was calculated as 100 × (experimental release − spontaneous release)/(maximun release − spontaneous release). Target cells incubated in medium alone and with medium plus 5% Triton X-100 were used to determine spontaneous and maximum chromium release, respectively.

ELISA

ELISA was used to determine antibody responses against HIV-1_{IIIB}. Serum and faecal specimens were collected on day 28 after immunization. Sample blood was collected by retro-orbital puncture under anaesthesia with diethyl ether and the serum obtained was stored at 4°C until the antibody assay as described elsewhere [22]. Faecal extract samples were prepared by suspending 100 mg of faecal pellets in 1 ml of PBS. After centrifugation at 12 000 rev/min, the supernatant was collected and stored at −20°C until use. ELISA for HIV-1_{IIIB} was done according to the protocol already described [4, 22]. Ninety-six-well microplates (Falcon, NJ) were coated with HIV-1_{IIIB} V3 peptide-multiple antigenic peptide (MAP) at a concentration of 5 μg/ml. Serial dilutions of sera or faecal samples from immunized mice were added to the wells after blocking with 1% bovine serum albumin (BSA) in PBS and washing with PBS-T (0.05% Tween 20). Wells were then treated with peroxidase-labelled anti-mouse IgG, IgG1 or IgG2a (Organon Teknika, West Chester, PA) as the secondary antibody. The plates were coloured with *o*-phenylenediamine dihydrochloride (Wako Chemical, Osaka, Japan) and read at 490 nm on a plate reader. For estimation of secretory IgA (sIgA), antibody against the V3 peptide was also used. Antibody titres were expressed as the reciprocal of the final detectable dilution which gave an optical density (OD) at 490 nm of > 0.2 OD units above the pretreated control. The IgG1/IgG2a ratio was calculated from the reciprocal $\log_2$ titres.

Histopathological analysis

Two micrograms of pCMV160IIIB/REV formulated with 10 μg of MIP-1α plasmid dissolved in PBS were injected into the biceps femoral muscles of BALB/c mice. At 1, 3, 5, 7 and 14 days after injection, muscles were resected, fixed with 10% buffered formalin, and embedded in paraffin. Thin sections were then prepared and stained with haematoxylin and eosin for light microscopic observation. Negative control mice were injected with the same amount of DNA vaccine alone.

Statistical analysis

Statistical analyses for comparison of two groups were conducted using an unpaired two-tailed *t*-test or one-way factorial analysis of variance (ANOVA) for distribution parameters. Significance was defined as $P < 0.05$ in both analyses.

RESULTS

Go to: Choose Go

HIV-1-specific antibody responses

To examine dose-related immune responses to MIP-1α-mediated DNA vaccination, we administered the DNA vaccine with 1, 10 and 50 μg of MIP-1α expression plasmid by intramuscular and intranasal immunization. The HIV-1-specific serum IgG and faecal IgA antibodies were analysed by ELISA on day 28 (Table 1). Antibody responses were significantly enhanced when 10 μg of MIP-1α expression plasmid were administered together with the immunogen ($P < 0.05$). Therefore, this dose was used in subsequent assays to evaluate HIV-1-specific immunity, including analyses of immunoglobulin subclasses and histological examinations. Intramuscularly the DNA vaccine alone was able to induce a serum IgG antibody

pCAGGSMIP-1α. pCAGGS vector was digested with *Xho* I restri...

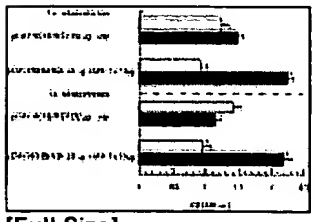

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Fig. 2. Immunoglobulin subclasses of HIV-specific antibody. BALB/c mice were given 2 μg of DNA vaccine...

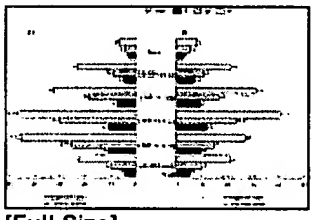

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Fig. 3. Cytotoxic T lymphocyte (CTL) activity of MIP-1α expression plasmid. (a) By intramuscular admi...

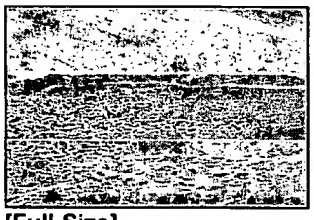

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Fig. 4. Histological examination of injected mice. (a) Injected with 2 μg of DNA vaccine alone. (b) In...

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titre of 2^6, and similar titres were observed when the adjuvants were included. Intranasal administration with the adjuvant similarly enhanced the IgG responses of the DNA vaccine. Intranasal administration was also effective in generating high levels of faecal IgA antibody. The antibody titres obtained with the DNA vaccine alone and with vaccine plus pCAGGS-empty were the same with either route, suggesting that the adjuvant effect was not caused by the pCAGGS expression vector. Mice serving as negative controls showed no detectable antibody response.

HIV-1-specific immunoglobulin subclass analysis

The vaccine-induced antibodies were then examined for their immunoglobulin subclass and titre using serum samples from the intramuscular and intranasal groups. As shown in Fig. 2, IgG2a titres increased and IgG1 titres decreased, causing a sizeable drop in the IgG1/IgG2a ratio, which was significantly lower than that obtained with inoculation of DNA vaccine alone in both routes, suggesting that a Th1-type response was induced by co-inoculation with the DNA vaccine and MIP-1α expression plasmid.

HIV-1-specific CMI responses

Dose-related DTH responses and CTL activity were also evaluated in both intramuscularly and intranasally immunized mice. HIV-1-specific DTH was analysed using the footpad swelling response 2 weeks after immunization (Table 2), and this response was found to be similar in both groups of mice. When the DNA vaccine was inoculated with 10 μg of the MIP-1α plasmid, the footpad swelling response was strongly enhanced compared with that of mice injected with the DNA vaccine alone. There was no significant difference between mice immunized with the DNA vaccine alone and with the vaccine plus pCAGGS-empty. Mice serving as negative controls showed no significant increase in the footpad swelling response.

Another experiment was conducted to determine whether co-inoculation of the DNA vaccine with the MIP-1α expression plasmid via the two routes could induce stronger CTL responses than inoculation of the immunogen alone. As shown in Fig. 3, strongly enhanced CTL activity was noted not only at an E:T ratio of 80 but also at a ratio of 20, when the vaccine was co-inoculated with 10 μg of the MIP-1α plasmid. There were no substantial differences between the two routes with respect to either HIV-1-specific DTH or CTL activity. The data of three other CTL experiments gave the same results, suggesting that the DNA vaccine with MIP-1α expression plasmid induced a higher level of HIV-1-specific CMI than did the vaccine alone.

Inflammatory cell accumulation in the MIP-1α-injected muscles

On histopathological examination, a substantial level of inflammatory cell infiltration composed of histocytes and lymphocytes was observed lasting from 1 to 7 days after injection. The maximal accumulation of lymphocytes in the injected muscles was observed 3 days after plasmid injection (Fig. 4b,c), indicating that strong inflammation was caused by MIP-1α injection. This was not observed in muscles injected with the DNA vaccine alone (Fig. 4a) and injected after 14 days. These data demonstrate that MIP-1α is a cytokine which displays chemoattractive activity for inflammatory cells.

DISCUSSION

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We previously reported that the use of expression plasmids for certain cytokines or costimulatory factors enhanced the immune responses induced with a DNA vaccine [7-10]. There have been several studies suggesting that DNA plasmids encoding a functional gene can elicit protective immunity in mice against certain pathogenic viruses [4, 23, 24]. Techniques employing immunological adjuvants together with plasmid DNA are not only simple to perform but also offer other several advantages including low cost, easy production, facilitated quality control, a continuous production of protein, and no risk of inadvertent infection. Our preliminary

experiments revealed that the half-life of IL-2 and IL-12 protein *in vivo* is short compared with their expression plasmid forms (unpublished data). Therefore, in an effort to develop more effective adjuvants, we considered MIP-1α co-inoculation with DNA vaccine to promote various forms of the immune response.

In this study, we examined the immune modifications elicited by MIP-1α expression plasmid. Although DNA vaccines without adjuvant are able to generate antigen-specific CMI, the faecal IgA antibodies were significantly activated and DTH response and CTL activity were consistently enhanced when the DNA vaccine was co-inoculated with MIP-1α either intramuscularly or intranasally (Table 2 and Fig. 3). Therefore, co-inoculating immunogenic DNA with MIP-1α augments the potency of DNA vaccinations. Moreover, the IgG1/IgG2a ratio was significantly lower than that obtained using the DNA vaccine alone, suggesting activation of Th1-type cells (Fig. 2). Activation of a Th1-type immune response is vital for HIV-1 protection or therapeutic efficacy. In murine models, cytokines such as IL-2 and IFN-γ produced by Th1 cells support the development of cellular immunity, including the CTL response and production of the IgG2a immunoglobulin isotype. Cytokines such as IL-4 and IL-5 produced by Th2 cells promote B cell activation and immunoglobulin class switching processes, which are typified by a predominance of the IgG1 immunoglobulin isotype [25, 26]. The relative predominance of IgG1 over IgG2a or *vice versa* can be influenced by the method of DNA inoculation (gene gun *versus* saline injection) as well as by the form of expressed antigen (membrane-bound or secreted) [27, 28]. We used MIP-1α because of its known activation of macrophages and its ability to induce IFN-γ production [29]. Activation of Th1-derived immunity elicited a DTH response, CTL activity, and IgG2a antibody production, and our results accommodate this. Recently, three murine CC-chemokine (MIP-1α, MIP-1β, RANTES) receptors which display different forms or chemokine binding have been described [30, 31]. The chemokine ligands for these receptors provide signalling in T cells and deliver different intracellular signals that activate a Th1 response and enhance IFN-γ production. In the present study, we did not obtain direct data about *in vivo* efficacy of MIP-1α expression plasmids because of the limitations of HIV-1 infection in mice.

We also demonstrated that injection with MIP-1α caused massive inflammatory infiltration in the injected site (Fig. 4). Histopathological examination demonstrated that MIP-1α has chemoattractive activity, which is consistent with previous studies [7]. In addition to adjuvant activity, MIP-1α suppresses the growth of HIV, possibly by interfering with HIV binding to CC-CKR3 and CC-CKR5 [30, 31]. Molecular identification of these fusion cofactors is critical for understanding the pathogenesis of HIV-1 infection and would be useful for designing therapeutic strategies. MIP-1α also plays an important role *in vivo* in the development of both acute and relapsing experimental autoimmune encephalomyelitis (EAE) [32]. In support of a vital role for MIP-1α in Th1-mediated inflammatory disease, Cook and others have shown recently that MIP-1α gene-deficient mice fail to develop virus-induced autoimmune heart disease [33]. These reports along with the data of the present study emphasize the importance of CC chemokine selectivity during the inflammatory immune response in terms of their roles as mediators of chemotaxis to the Th1 subtype.

Including the CpG motif in DNA vaccines as an adjuvant may increase humoral and cellular immunity to a weakly antigenic protein β-galactosidase encoded by it or a co-injected plasmid [34]. There are reports that CpG-based DNA sequences in the plasmid backbone of DNA vaccines promote antigen-specific immunity [35, 36]. To eliminate the promoter region function as a confusing variable, we inoculated MIP-1α-free pCAGGS with the DNA vaccine using both routes of immunization (Tables 1 and 2). The antibody titres and the DTH response were not significantly different from those obtained using the DNA vaccine alone, suggesting the adjuvant activity of MIP-1α was not due to the CpG motif.

DNA vaccine therapy is thought to be an effective method for treating patients with AIDS. Intramuscular injection has been favoured for eliciting immune response over the past years because of its many advantages [7]. Recent reports have shown that intranasal administration of DNA vaccine induces strong antibody production, particularly production of mucosal sIgA antibody, and a high level of HIV-specific CTL activity [37-39]. We found in the present study that the DTH response and

CTL activity were significantly enhanced when the DNA vaccine was inoculated intranasally with MIP-1α expression plasmid (Table 2; Fig. 3b). A high level of HIV-specific mucosal sIgA antibody was also observed when we used this route of administration. Compared with the intramuscular route, intranasal administration of DNA vaccine is safe, easily carried out, and has f wer side-effects. However, further detailed analysis is necessary to evaluate this method fully. Taking all data together, we consider that the present approach of formulating adjuvants for use with plasmid DNA and administration via the intranasal route is the simplest and most economical method for providing immunity against this disease.

In conclusion, our present findings clearly show that DNA vaccine co-inoculated with MIP-1α expression plasmid induces a substantial level of HIV-specific CMI and that similar vaccine-plasmid combinations may be useful for designing therapeutic strategies to combat HIV infection.

Acknowledgements Go to: Choose Go

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Up-regulation of β-chemokines and down-modulation of CCR5 co-receptors inhibit simian immunodeficiency virus transmission in non-human primates

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SUMMARY

A non-cognate mechanism of protection against human immunodeficiency virus-1 (HIV-1) infection involves up-regulation of β-chemokines, which bind and may down-modulate the CCR5 co-receptors, thereby preventing transmission of M-tropic HIV-1. The objective of this investigation was to evaluate this mechanism *in vivo* in non-human primates. Rhesus macaques were immunized by a modified targeted lymph nodes (TLN) route with recombinant simian immunodeficiency virus (SIV) glycoprotein 120 (gp120) and p27 in alum, and adsorbed recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) with either interleukin (IL)-2 or IL-4. Immunization induced significant increases in the concentrations of CD8 cell-derived suppressor factor (CD8-SF), regulated on activation normal T cells expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β, and down-modulation of the proportion of cells expressing CCR5 ($r = 0{\cdot}737$, $P < 0{\cdot}05$). The macaques were then challenged with SIVmac 220 by the rectal mucosal route. The plasma SIVmac RNA showed a significant inverse correlation with the CD8-SF or the concentration of the three β-chemokines ($r = 0{\cdot}831$ and $0{\cdot}824$, $P < 0{\cdot}01$), but a positive correlation between the proportion of $CCR5^+$ cells and SIVmac RNA ($r = 0{\cdot}613$, $P = 0{\cdot}05$). These results demonstrate for the first time *in vivo* that immunization up-regulates β-chemokines, which may down-modulate CCR5 co-receptors, and both functions are significantly correlated with the viral load. Hence, the non-cognate β-chemokine–CCR5 mechanism should be considered as complementary to specific immunity in vaccination against HIV.

INTRODUCTION

Mucosal human immunodeficiency virus (HIV) infection has been responsible for the predominantly heterosexual transmission in developing countries and homosexual transmission in developed countries. The genital or rectal mucosa and the draining regional lymph nodes are the primary and secondary barriers that the virus has to breach.[1–3] One approach to the prevention of mucosal infection has been direct vaginal[4,5] or rectal[6,7] immunization with simian immunodefiency virus (SIV) antigens, but this has not achieved consistent protection. An alternative approach has been to target the iliac lymph nodes, which function as an inductive immune site for the genital and rectal mucosa.[3] Immunization by the latter route with the recombinant SIV subunit envelope glycoprotein 120 (gp120) and core p27 antigens in alum resulted in either total protection or a significant decrease in viral load after challenge with a pathogenic SIV.[8] The mechanism of protection was not clarified, but in addition to SIV-specific serum immunoglobulin G (IgG) and secretory immunoglobulin A (IgA) antibodies, and $CD4^+$ T-cell proliferative responses, IgA antibody-forming B cells were demonstrated in the regional iliac lymph nodes. The novel and significant finding was an increase in secretion of the CD8-suppressor factor (CD8-SF) and the three β-chemokines – regulated on activation normal T cells expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β – derived from the regional lymph nodes and peripheral blood $CD8^+$ cells, as compared with immunized controls.[8] These results suggested that in

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Abbreviations: MFI, median fluorescence intensity; s.c., subcutaneous; SEM, standard error of the mean; SF, suppressor factor; sIgA, secretory immunoglobulin A; SIV, simian immunodeficiency virus; TLN, targeted lymph node.

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addition to cognate SIV-specific immunity, immunization can elicit CD8-SF (or CAF)[9] and three β-chemokines,[10] which may inhibit SIV or HIV replication by blocking the CCR5 co-receptors or inhibiting SIV transcription. Furthermore, there is *in vitro* evidence that RANTES or stromal-derived factor (SDF-1) chemokine down-regulates the corresponding CCR5 or CXCR4 co-receptors, respectively.[11,12] These receptors are internalized within 20 min but are recycled to the cell surface during the next 20 min. If immunization up-regulated the concentration of β-chemokines, cell surface expression of CCR5 might be down-modulated *in vivo*. Indeed, alloimmunization in humans up-regulated the three β-chemokines and down-modulated the CCR5 co-receptors and, interestingly, only the latter was maintained for up to 1 year.[13]

This investigation was based on the hypothesis that immunization with SIV antigens, with or without cytokines, *in vivo* may, in addition to specific immunity to SIV, up-regulate CD8-SF and elicit innate immune responses by generating β-chemokines that block and down-modulate CCR5, thereby decreasing SIV transmission. In this experiment we targeted for the first time the readily accessible subcutaneous (s.c.) inguinal and external iliac lymph nodes, instead of the deep internal iliac lymph nodes, in an attempt to avoid the deep injection that may not be acceptable for use in humans. The rationale was to induce immune responses at the mucosal site of entry of SIV, in the draining lymph nodes and the circulation, in order to generate three immune barriers to the virus.

MATERIALS AND METHODS

Immunization of macaques

Nine mature macaques were immunized by a modified targeted lymph node (TLN) route, which involved conventional s.c. injection, but given in the inguinal region. The vaccine was administered s.c. three times at two sites, near the inguinal and external iliac lymph nodes on both sides. Immunization was carried out at approximately monthly intervals, and a fourth booster injection was given into the gluteal muscles. A group of three macaques was immunized with recombinant SIVgp120 and p27 (200 μg of each) in alum (AluGel; Uniscience, London, UK). Recombinant SIVmac 251 gp120 was expressed in Baculovirus-infected cells,[14] and rSIVp27 was expressed in pGEX-3X as a glutathione-S-transferase fusion protein. A second group of three macaques was given the same vaccine but mixed with recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (5 μg/kg; Sandoz Pharmaceuticals, Surrey, UK) and simian interleukin (IL)-2 (5 μg/kg of each).[15] The third group was given the vaccine with GM-CSF and simian IL-4 (4 μg/kg). All experimental procedures were performed under sedation with ketamine hydrochloride (10 mg/kg) and Domitor (1 mg/kg).

Serum and rectal fluid antibody assays

Samples of fluid were collected before and at monthly intervals after each immunization. Blood collected from the femoral vessels was defibrinated and the serum separated. A constant volume of ≈ 6·0 ml of rectal washings was collected without trauma, with the aid of flexible, lubricated paediatric nasogastric tubes, as described previously.[6] IgA and IgG antibodies to gp120 and p27 were determined by enzyme-linked immunosorbent assay (ELISA), as described previously.[6] Briefly, plates were coated with a predetermined optimal concentration of gp120 or p27 (at 1 μg/ml) and with a random peptide 20^{mer} (R20) as a control antigen; they were incubated with doubling dilutions of the test samples. Bound antibody was detected by incubation with rabbit IgG anti-monkey IgA (8 μg/ml; Nordic Immunological Labs, Tilbury, The Netherlands) or IgG (2 μg; Sigma Fine Chemicals Ltd, Poole, Dorset, UK), followed by incubation with affinity-purified goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma Fine Chemicals Ltd). For rectal washings (concentrated ×4), the starting samples were used neat and then diluted 1 : 2, 1 : 4, etc., whereas serum samples were diluted 1 : 100, 1 : 200, etc. Rectal washings were concentrated by lyophilization and the concentration step was taken into account when the results were calculated.

T-cell proliferative responses

T-cell cultures were set up by separating mononuclear cells from defibrinated blood, using Lymphoprep (NYCOMED, Oslo, Norway) density-gradient centrifugation, before and after immunization of all macaques.[3] The cells were cultured without antigen and with 1 and 10 μg/ml of gp120, p27, a control peptide (R20) or concanavalin A (Con A) in 96-well round-bottomed plates (Costar, Cambridge, MA), containing RPMI-1640 (Gibco, Paisley, Strathclyde, UK). The results were expressed as stimulation indices (ratio of counts with and without antigen), for cultures stimulated with the optimum (10 μg/ml) gp120 or p27 concentration. All cultures yielded high stimulation indices and counts with Con A, and no significant increases in counts were seen with the control peptide (data not shown).

Assay of CD8-SF and β-chemokines in stimulated culture supernatant

Peripheral blood mononuclear cells (PBMC) were separated and $CD8^+$ cells were enriched by depletion of $CD4^+$ cells, B cells and monocytes by panning with CD4 (OKT4 hybridoma culture supernatant) and antibodies to human immunoglobulin (Serotec, Oxford, UK). The enriched $CD8^+$ cells were then cultured with 10 μg/ml of phytohaemagglutinin (PHA) for 3 days. Preparation of the CD8 culture supernatant was then carried out according to a method described previously.[8,9] PHA-stimulated $CD8^+$ cells were cultured at a concentration of 3×10^6 cells/ml in RPMI-1640 containing 10% fetal calf serum (FCS) and 10% human IL-2 preparation (TLF; Biotest, Solihull, UK). After 2 days of incubation (at 37°, in 5% CO_2), the culture supernatant was collected, and the cells were reconstituted with fresh medium; this was repeated up to three times. At each passage the cellular density was adjusted to 3×10^6 cells/ml. The collected supernatants were filtered through a 0·45-μm filter and stored at −70° for CD8-SF and the β-chemokine assay. SIVmac acutely infected $CD4^+$ cells were cultured in the presence of CD8-SF at 1 : 2 and 1 : 5 dilutions in 96-well plates. The CD8-SF were replenished every 2–3 days and on day 7 the culture supernatant was used to determine the reverse transcription (RT) activity by using the Quant RT kit (Amersham, UK). RANTES, MIP-1α, MIP-1β and macrophage chemoattractant protein-1 (MCP-1) were assayed in the $CD8^+$ cell culture supernatant using a specific

Table 1. The modulating effect of granulocyte–macrophage colony-stimulating factor (GM-CSF) with interleukin (IL)-2 or IL-4 on subcutaneous (s.c.) inguinal and external iliac targeted lymph node (TLN) immunization with simian immunodeficiency virus (SIV) glycoprotein 120 (gp120) and protein 27 (p27) in alum (×4)

	Targeted inguinal + external iliac LN							
	Vaccine (SIV gp120 + p27)		Vaccine + GM-CSF + IL-4		Vaccine + GM-CSF + IL-2		*Targeted iliac LN vaccine	
Immune response	Pre†	Post†	Pre	Post	Pre	Post	Pre	Post
Serum IgG to gp120	<50	147200 (131317)	<50	**409600** (0)	<50	409600 (0)	<50	7467 (2822)
Serum IgA to gp120	<50	8553 (2153)	<50	68267 (17067)	<50	**341333** (68267)	<50	4800 (1600)
Serum IgG to p27	<50	55467 (25953)	<50	546133 (136533)	<50	**682667** (136533)	<50	12800 (6400)
Serum IgA to p27	<50	8533 (2133)	<50	136533 (34133)	<50	**341333** (68267)	<50	3200 (1600)
Rectal sIgA to gp120	<0.5	24.7 (19.7)	<0.5	0.5 (0.3)	<0.5	**53.3** (37.3)	<0.5	4 (0)
Rectal IgG to gp120	<0.5	6.0 (2.0)	<0.5	0.5 (0.3)	<0.5	**6.7** (1.3)	<0.5	4 (0)
Rectal sIgA to p27	<0.5	66.7 (34.7)	<0.5	0.5 (0.3)	<0.5	**96** (80)	<0.5	4 (0)
Rectal IgG to p27	<0.5	6.7 (1.3)	<0.5	0.5 (0.3)	<0.5	**21.3** (5.3)	<0.5	3 (1)

No serum (<1 : 50) or rectal (<1 : 0·5) antibodies were detectable before immunization. Mean titres (± SEM) of serum and rectal antibodies are given for the four groups, each comprising three macaques.

*The data of macaques immunized by the deep internal and external iliac TLN route are presented, for comparative purposes, from a previous publication.[8]

†Pre and post refer to pre- and postimmunization titres, respectively.

IgA, immunoglobulin A; IgG, immunoglobulin G; sIgA, secretory immunoglobulin A.

enzyme immunoassay (R & D Systems Europe Ltd, Abingdon, Oxfordshire, UK).

Cell surface expression of CCR5 by flow cytometry

Freshly isolated PBMC were incubated with monoclonal antibodies (mAb) to CCR5 (227), kindly supplied by Dr P. Gray (ICOS Corporation, Seattle, WA). The cells were incubated with fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse IgG (Dako, Glostrup, Denmark), or with the latter alone as a control, and flow cytometry was performed using a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Franklin Lakes, NJ) running LYSIS II software for both acquisition and analysis.

Rectal mucosal challenge with SIV

Viral challenge was carried out by topical application to the rectal mucosa of 25MID50 (median monkey infectious dose) of SIVmac 220, which is a cell-free virus stock prepared from the spleen of a rhesus monkey infected with the J5 molecular clone of SIVmac 251 (32H).[16]

Determination of plasma SIVmac RNA and cell-associated SIV load

The plasma concentration of SIVmac RNA was determined using the SIVmac branched DNA assay of Chiron Diagnostics (Amsterdam, the Netherlands) and expressed in equivalents/ml (Eq/ml) (one equivalent is approximately one molecule of SIVmac RNA). PBMC-associated virus loads were determined by limiting-dilution analysis. Briefly, simian PBMCs were separated from whole blood by centrifugation on Ficoll Paque (Pharmacia Biotech, Bucks, UK). Cells were diluted from 10^6 to 4×10^5 and subsequently in fivefold steps to 130 cells, and duplicate cultures were co-cultured with the human T-cell line C8166 in 25-cm^2 flasks. Medium and C8166 cells were replenished every 3–4 days, and the total culture volume was maintained at ≈ 15 ml. All cultures were kept for 30 days or until a cytopathic effect was apparent. Virus isolation was confirmed by indirect immunofluorescence using polyclonal anti-SIV serum. Fifty per cent end-points were calculated using the Karber formula, and the results were expressed as the number of infected cells per 10^6 PBMCs. In addition, the polymerase chain reaction (PCR) was carried out in all macaques, as described previously.[17]

Statistical analysis

Data from the three groups of macaques were expressed as mean ± SEM. Any relationship between the β-chemokines and CCR5 or plasma viral load, or between CCR5 or CD8-SF and the plasma viral load, was determined by calculating the correlation coefficient. The Student's *t*-test was used to analyse the differences between the percentage and median fluorescence intensity (MFI) of CCR5 in immunized and unimmunized macaques.

RESULTS

Serum antibodies to SIVgp120 and p27

Serum IgG antibody titre, and to a lesser extent IgA and secretory IgA (sIgA) antibody titres, to SIVgp120 and p27 were higher after the s.c. inguinal and external iliac immunization than after the deep internal iliac TLN route of immunization, which was added for comparison but was published previously[8] (Table 1). Immunomodulation with GM-CSF and either IL-2 or IL-4 elicited an increase in serum IgG antibody titres to both SIV antigens, and surprisingly no significant variation was observed between the macaques, compared with those given the vaccine alone. However, higher IgA antibody titres to gp120 and p27 were induced with the vaccine containing GM-CSF + IL-2, compared with GM-CSF + IL-4. These results suggest that targeting the s.c. inguinal and external iliac lymph nodes with SIVgp120 and p27 in alum induces higher titres of serum antibodies than those achieved by the deep injection targeting the internal iliac lymph nodes. The antibody titres

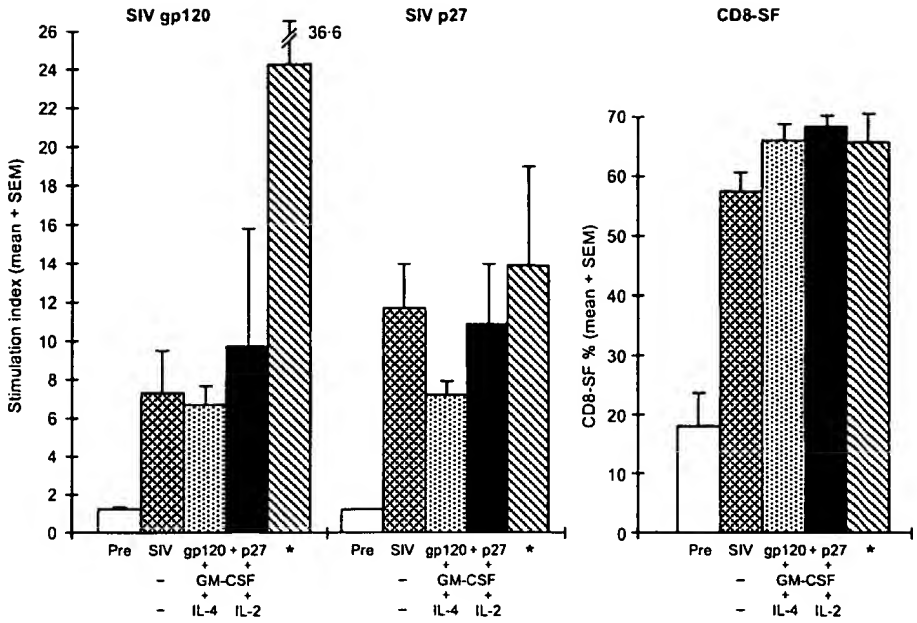

Figure 1. T-cell proliferative responses to simian immunodeficiency virus (SIV) glycoprotein 120 (gp120) and protein 27 (p27) and CD8-suppressor factor (CD8-SF) before (Pre) and after subcutaneous (s.c.) inguinal and external iliac* targeted lymph node (TLN) immunization (×4) with SIVgp120 and p27 in alum, with or without granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4 or IL-2. The results are presented as mean (+SEM) of the stimulation indices. The data of three macaques immunized by the deep targeted iliac lymph node route are presented for comparison.[8]

could be further enhanced by adding GM-CSF with either IL-2 or IL-4. Correlations between the plasma SIVmac RNA and serum IgG or IgA antibodies to SIVgp120 and p27 were calculated but none of the correlation coefficients reached the 5% level of significance ($r = 0{\cdot}476$, 0·411, 0·500, 0·478, respectively). However, the variation in titres between the outbred macaques was considerable, and this was found especially with IgG antibodies to SIVp27. Neutralizing antibodies were examined by Dr D. Montefiore (Duke University, Medical Centre, Durham, NC) but no significant antibodies were detected in these macaques.

Rectal mucosal antibodies to SIVgp120 and p27

sIgA and IgG antibodies to SIVgp120 and p27 were raised after immunization with SIVgp120 and p27 in alum (Table 1). A further increase in titres of these antibodies was found when GM-CSF and IL-2 were adsorbed to alum and added to the vaccine, but the variation in sIgA antibody titres between individual macaques was considerable. Surprisingly, adsorption of GM-CSF and IL-4 to alum inhibited both sIgA and IgG rectal antibodies, unlike the increase in serum antibodies (Table 1). However, SC inguinal and external iliac TLN immunization with SIVgp120 and p27 in alum, with or without GM-CSF and IL-2 elicited at least as high sIgA and IgG antibody levels as those found after the deep internal iliac TLN route of immunization. Calculations of the correlation coefficients between the rectal sIgA or IgG antibodies to SIVgp120 or p27 and the plasma SIV RNA failed to yield significant correlation ($r = 0{\cdot}191$ and 0·163).

T-cell proliferation to SIVgp120 and p27

The T-cell proliferative responses to SIVgp120 and p27 were raised in all nine macaques from a stimulatory index of $<2{\cdot}0$ before to a mean (± SEM) of between 6·7 ± 0·9 and 11·8 ± 2·7 after immunization, but there was no significant difference between the three groups of SC-TLN-immunized macaques (Fig. 1). However, the T-cell proliferative response stimulated by SIVgp120 was much lower than that elicited by the deep iliac TLN immunization (24·4 ± 12·2).

CD8 cell-generated suppressor factor

The CD8 cell-generated SF showed an increase from 17·7 ± 5·8% before immunization to 57·5 ± 3·1% after immunization with SIVgp120 and p27 (Fig. 1). There was a further slight increase with the addition of either M-CSF + IL-4 (65·9 ± 2·9%) or GM-CSF + IL-2 (68·9 ± 2·0%). These results were similar to those induced by targeting the internal and external iliac lymph nodes with the same vaccine but without any cytokines (Fig. 1).

CD8-cell generated β-chemokines

The $CD8^+$ T-cell generated factors showed a significant increase in the concentrations of RANTES, MIP-1α and MIP-1β after immunization with SIVgp120 and p27 in alum (Fig. 2). Addition of GM-CSF with either IL-2 or IL-4 to the vaccine increased slightly the concentration of each of the three β-chemokines, without any obvious differential effect on these chemokines, except for an increase in RANTES of the GM-CSF and IL-2-treated animals. These results were consistent

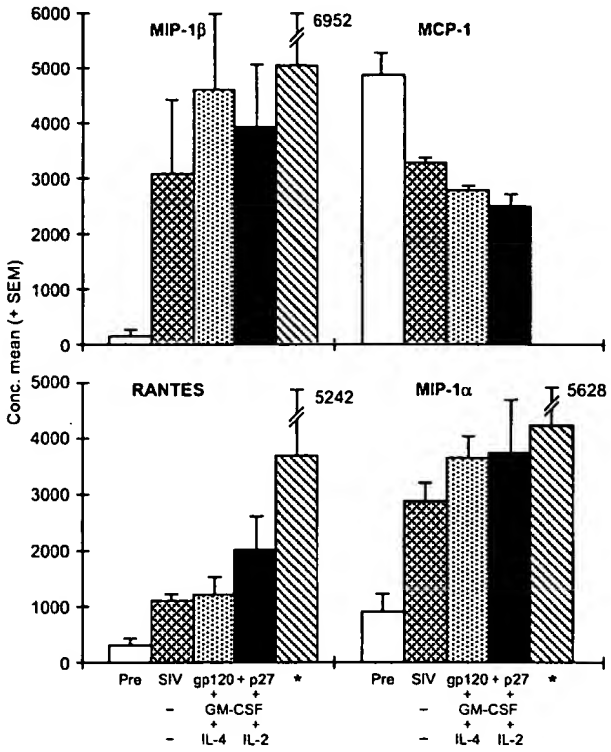

Figure 2. Concentrations of RANTES, MIP-1α, MIP-1β and MCP-1 (mean ± SEM pg/ml), before and after subcutaneous (s.c.) inguinal and external iliac* targeted lymph node (TLN) immunization (×4) with simian immunodeficiency virus (SIV) glycoprotein 120 (gp120) and protein 27 (p27) in alum alone or with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-2 or IL-4. The chemokines were assayed in phytohaemagglutinin (PHA)-stimulated $CD8^+$ cell culture supernatants ≈ 1 month after the fourth immunization. The data of three macaques immunized by the deep TLN iliac route are presented for comparison.[8] Pre, chemokine concentration prior to immunization.

with those obtained previously by the deep TLN immunization, except for RANTES, the concentration of which was greater (6·2×), although the level of unimmunized macaques for that series was higher at 602 ± 339 pg/ml. Surprisingly, there was a decrease in the concentration of MCP-1 after immunization, which was slightly more marked with the cytokine immunomodulators. The combined concentration of RANTES, MIP-1α and MIP-1β showed a progressive increase in concentration after each of the four immunizations (Fig. 3). The concentration was highest (9997 ± 3303 pg/ml) when the vaccine was administered by the s.c. TLN route with GM-CSF+IL-2 but, owing to variation in concentrations of the chemokines and the small number of macaques used, these differences failed to reach the 5% level of significance. The concentration of the three β-chemokines generated by the deep iliac TLN immunization was higher (13 096 ± 5768) than that resulting from the s.c. inguinal TLN immunization with GM-CSF+IL-2 (9997 ± 3003).

Cell-surface expression of CCR5

Flow cytometry examination of CCR5 directly on freshly isolated PBMC showed a decreased proportion and cell surface expression of CCR5 in PBMC of immunized macaques, which reached the lowest levels in those given SIVgp120 and p27 with GM-CSF+IL-2 (Fig. 4a). Unimmunized macaques showed 29·0 ± 2·3% $CCR5^+$ cells and MFI of 209 ± 25·1, which decreased to 25 ± 6·7% and MFI 39·9 ± 4·8 after immunization with gp120 and p27 (Fig. 4b). A further decrease to 20·6 ± 2·8% and 17·1 ± 4·2% of $CCR5^+$ cells was found in the PBMC of macaques immunized with the vaccine and GM-CSF+IL-4 or IL-2, respectively, although the MFI showed little change (47·6 ± 7·6 and 42·8 ± 1·0, respectively). The proportion ($t=3·72$, $P<0·05$) and especially MFI ($t=6·20$, $P<0·001$) of CCR5 was significantly lower after, than before, immunization. Furthermore, a significant inverse correlation was found (Fig. 5a) between the total concentration of the three CD8 cell-generated β-chemokines (RANTES, MIP-1α and MIP-1β) and that of the percentage, but not the MFI, of CCR5 in PBMC after the last immunization ($r=0·737$, $P<0·05$, d.f. 7). Thus, the higher the concentration of the three β-chemokines, the lower the proportion of $CCR5^+$ cells, suggesting that the increased concentration of β-chemokines *in vivo* down-modulates the CCR5 cell-surface expression, as found previously *in vitro*.[11,12]

Plasma SIVmac RNA

Comparison of the plasma RNA load with the cell-associated SIV assay, 2 weeks after mucosal challenge, showed a significant correlation between the two assays ($r=0·71$, d.f. 7, $P<0·05$; data not presented). One of the three macaques

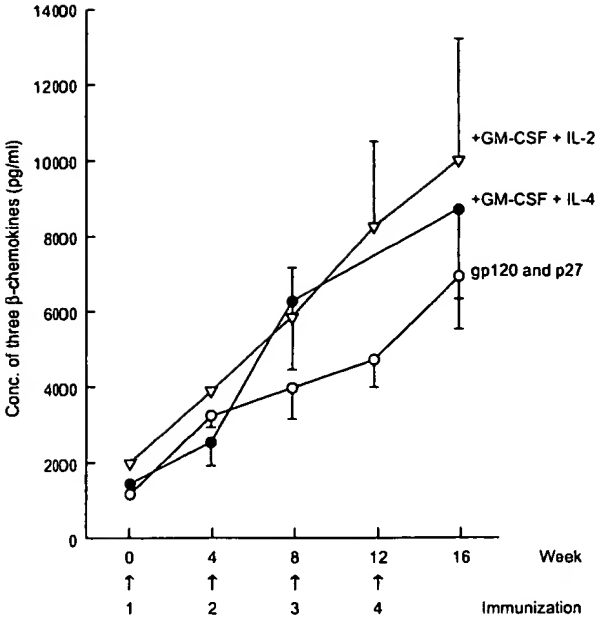

Figure 3. Sequential changes of the combined concentrations of RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β after subcutaneous (s.c.) inguinal and external iliac targeted lymph node (TLN) immunization with simian immunodeficiency virus (SIV) glycoprotein 120 (gp120) and protein 27 (p27) alone in alum or with granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4 or IL-2. The results are presented as mean (± SEM) before and 4 weeks after each of the four immunizations.

immunized with SIVgp120, p27 and GM-CSF with IL-2 adsorbed to alum was not infected after rectal mucosal challenge with SIVmac 220 and was also negative by the co-culture technique and by PCR. Although the remaining eight macaques were infected, a significant, inverse correlation (Fig. 5b) was found between the plasma SIVmac RNA and the CD8-SF ($r=0{\cdot}831$, d.f. 7, $P<0{\cdot}01$), as well as the concentration of the CD8 cell-generated three β-chemokines ($r=0{\cdot}824$, $P<0{\cdot}01$; Fig. 5c). The control β-chemokine (MCP-1) and five cytokines (IL-2, interferon-γ [IFN-γ], IL-12, IL-4 and IL-10) showed no correlation with the plasma SIV RNA (data not presented). In contrast, the percentage of $CCR5^+$ cells showed a positive correlation with the plasma SIVmac RNA ($r=0{\cdot}715$, $P=0{\cdot}05$; Fig. 5d). Thus, the higher the β-chemokine concentration, the lower the cell-surface expression of CCR5 and the lower the plasma concentration of SIVmac RNA. There was no significant difference between the nine macaques immunized with or without cytokines, although the group given GM-CSF + IL-2 yielded the lowest plasma SIV RNA load.

DISCUSSION

In view of the consistency of the immune responses and significant protection elicited by TLN immunization with recombinant SIVgp120 and p27 in alum,[8] we immunized nine macaques with the same vaccine. However, in order to avoid the deep injection used previously for the TLN immunization, which is unlikely to be suitable for use in humans, we modified the administration of the vaccine, giving it by s.c. injection into the region of the inguinal and external iliac lymph nodes. In addition, we attempted to modulate the immune responses by incorporating, in the SIVgp120 and p27 vaccine, GM-CSF with either IL-4 or IL-2, which were co-adsorbed to alum, in order to up-regulate T helper (Th)1 or Th2 responses.[18–20]

Whereas the deep TLN immunization elicited the highest $CD4^+$ T-cell stimulation and CD8 cell-derived β-chemokines, the s.c. inguinal and external iliac TLN immunization elicited higher serum and possibly rectal fluid antibody titres than the previous deep TLN immunization. There is no obvious explanation to account for this remarkable difference, but differential activation of the two groups of lymph nodes should be considered, corresponding to the predominantly mucosal drainage of a presumably high load of resident micro-organisms to the internal iliac, and that of cutaneous and mucosal drainage probably with a lower load of resident bacteria to the inguinal and external iliac lymph nodes. Surprisingly, immunomodulation with GM-CSF + IL-2 (a Th1 cytokine) up-regulated serum and sIgA to gp120 and p27, whereas GM-CSF + IL-4 (a Th2 cytokine) inhibited secretory IgA antibodies in the rectal fluid (Table 1). The mechanism of this inhibitory effect of IL-4 on sIgA is not understood and requires further investigation. The $CD4^+$ T-cell proliferative responses showed no significant differences in the macaques co-immunized with the cytokines but were lower than those elicited by the deep TLN route of immunization.

A decrease in the proportion (%) of PBMC expressing cell-surface CCR5 was noted in the immunized macaques ($P<0{\cdot}05$) but a much more significant decrease was found in the cell-surface expression (MFI) of CCR5 ($P<0{\cdot}001$). As GM-CSF is capable of down-modulating CCR5 and decreasing the capacity to support HIV entry,[21] this cytokine might have been involved in the two groups of macaques, treated with GM-CSF, which also showed the lowest proportion of $CCR5^+$ cells. However this is debatable, as it was administered only at the time of each immunization, the last of which was ≈ 2 weeks before challenge with SIV. We have also not excluded the possibility that SIVgp120 might bind and down-modulate CCR5, although there is no published evidence of this.

A further finding of this investigation was the significant inverse correlation between the concentration of three CD8 cell-generated β-chemokines (RANTES, MIP-1α and MIP-1β) and the proportion of CCR5 in PBMC after the last immunization ($P<0{\cdot}01$). These *in vivo* results suggest that immunization of macaques with SIVgp120 and p27 in alum up-regulates the three β-chemokines, which may down-modulate the cell-surface expression of CCR5 receptors – this is consistent with the *in vitro* data.[11,12] Furthermore, a significant, inverse correlation between the plasma SIVmac RNA and the three β-chemokines and a positive correlation between the SIVmac RNA and the proportion of $CCR5^+$ cells is the first *in vivo* evidence to support the β-chemokine–CCR5 interaction protecting SIV mucosal infection. We suggest that up-regulation of the three β-chemokines *in vivo* down-modulates the cell-surface expression (MFI) of CCR5 and decreases the proportion (%) of CCR5-detectable cells, both of which affect SIV transmission, resulting in an inverse correlation between the viral load and proportion of cells that carry a lower cell surface expression of CCR5. It should be noted that *in vitro* inhibition studies of SIV replication suggest that the concentration of the three relevant β-chemokines must be between 2·5

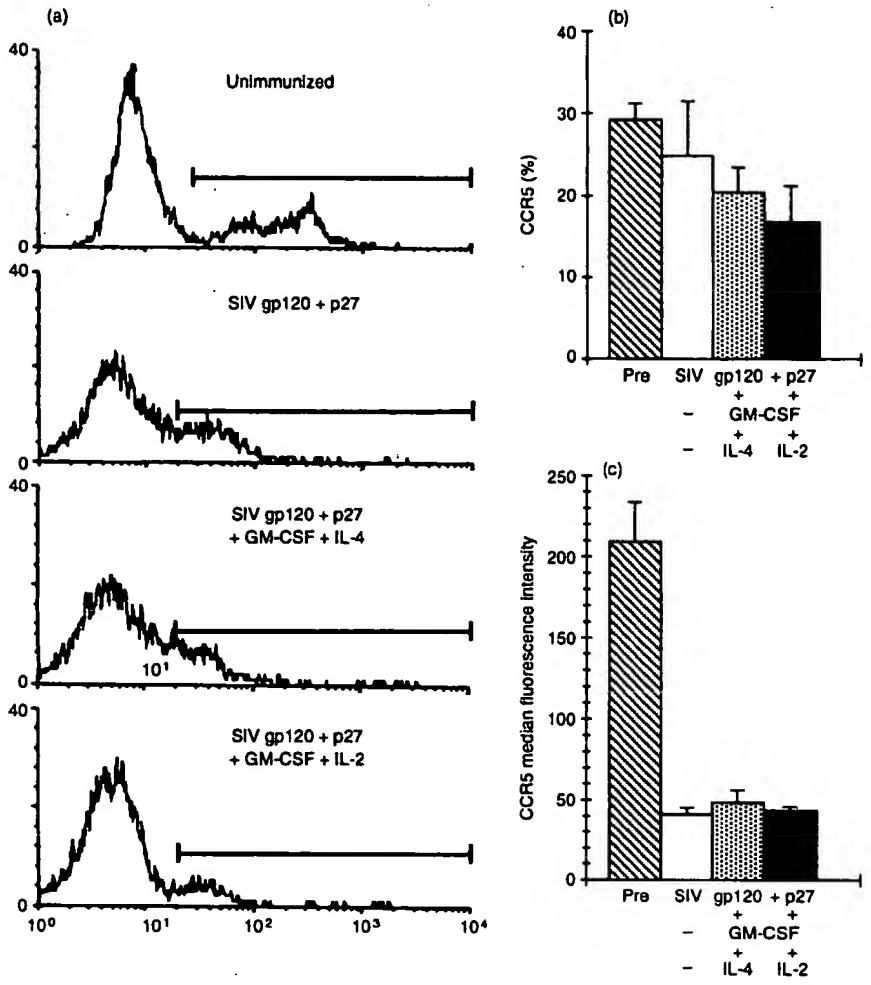

Figure 4. (a) Flow cytometry of representative results of the proportion and cell surface expression of CCR5 in an unimmunized macaque (32·1%, median fluorescence intensity [MFI] 298), and in macaques immunized with simian immunodeficiency virus (SIV) glycoprotein 120 (gp120) and protein 27 (p27) (25·3%, MFI 66·4), SIVgp120 and p27 + granulocyte–macrophage colony-stimulating factor (GM-CSF) + interleukin (IL)-4 (20·6%, MFI 103), and SIVgp120 and p27 + GM-CSF + IL-2 (11·4%, MFI 36). The results of the mean (± SEM) of each group of three macaques is given in (b) as the proportion of cells (%) and in (c) as the MFI of cell surface-expressed CCR5. Maximum decrease in percentage and MFI of CCR5 was found with macaques immunized with the vaccine containing GM-CSF + IL-2. Pre, chemokine concentration prior to immunization.

and 25 ng/ml to achieve 90–95% inhibition of SIV replication.[22] It is unlikely that these high concentrations will be required *in vivo*, in view of the proximity of CD8 and CD4 cells; nevertheless, concentrations of the three β-chemokines up to 13 ng/ml were reached. Indeed, high concentrations of RANTES (> 1000 ng/ml), but not MIP-1α or MIP-1β, can enhance the infectivity of M-tropic HIV.[23]

The possibility has to be considered that the redundancy in chemokine receptors to which SIV can bind, such as Bonzo (STRL), Bob (GPR15) or GPR1,[24–27] may enable the virus to adapt under the immune and chemokine pressures and infect the cells via an alternative co-receptor. However, subjects having the homozygous 32 bp deletion of CCR5, with a few exceptions, do not show evidence of co-receptor switch, as they remain free of HIV infection.[28–30] It is noteworthy that vaccination *per se* does not up-regulate the β-chemokines, as was found by immunization with measles virus in macaques[22] or influenza virus in humans.[31] Similarly, the adjuvant used (MDP, alum or ISCOM) did not affect the level of the β-chemokines.[22] However, using the same vaccine (SIVgp120 and p27) administered by different routes of immunization appears to affect the level of β-chemokine concentration.[8,22] Indeed, conventional intramuscular (i.m.) immunization up-regulates the concentration of the three β-chemokines, but the levels are five to eight times lower, and the CD8-SF is also lower, than those reached by the deep TLN route of immunization.[32] Furthermore, protection from rectal SIV challenge was found only in the deep TLN-immunized macaques, which was significantly associated with the levels of the three β-chemokines and CD8-SF, but not neutralizing antibodies.[8] We cannot exclude another possibility, that a decrease in the concentration of MCP-1 might also be involved, by attracting fewer macrophages and thereby decreasing the number of cells available for virus infection.

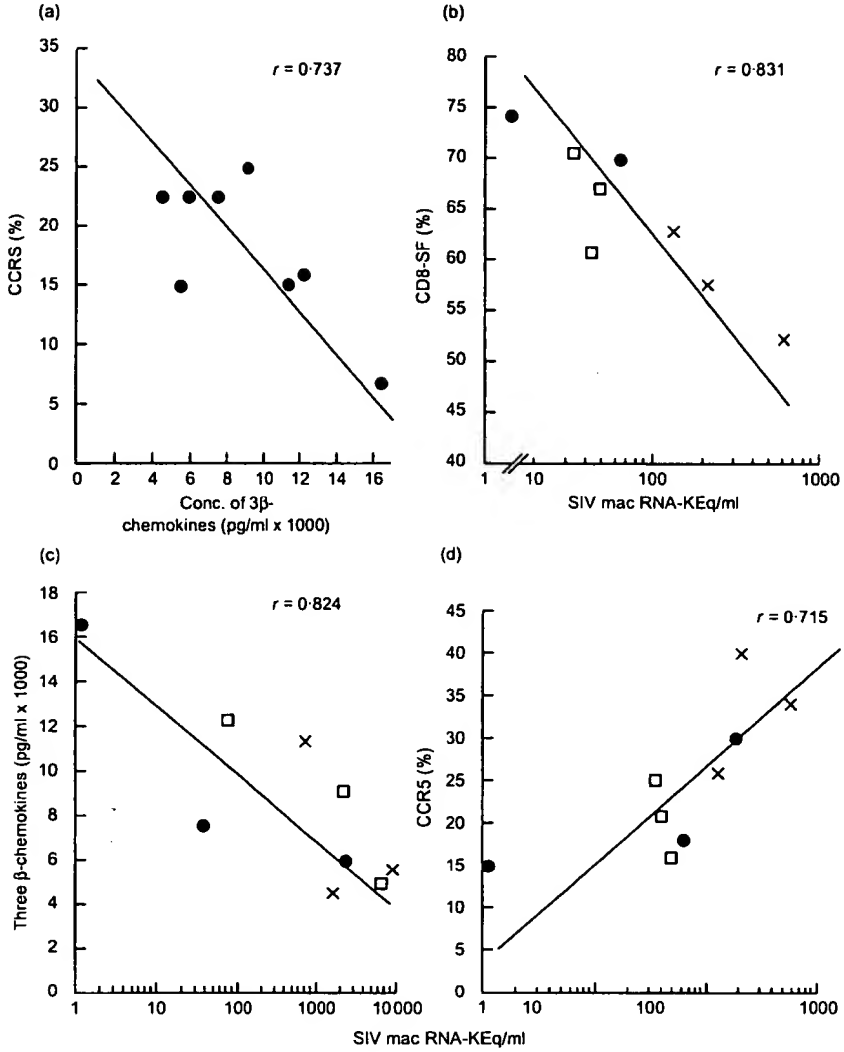

Figure 5 (a) Inverse correlation between the concentration of CD8-cell generated RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β, and the percentage of CCR5 co-receptors in peripheral blood mononuclear cells (PBMC) after the fourth immunization (before challenge) ($r = -0{\cdot}737$, d.f. 7, $P < 0{\cdot}05$). (b) Inverse correlation between CD8 cell-generated suppressor factor and the simian immunodeficiency virus (SIV)mac RNA 4 weeks after challenge with SIVmac 220 ($r = -0{\cdot}831$, d.f. 7, $P < 0{\cdot}01$). Correlation between the plasma SIVmac RNA 2 weeks after challenge with SIV (c) against the combined concentrations of the three β-chemokines ($r = -0{\cdot}824$, d.f. 7, $P < 0{\cdot}01$) and (d) against the percentage of $CCR5^+$ cells ($r = 0{\cdot}715$, d.f. 7, $P = 0{\cdot}05$), both after the last immunization. (×) glycoprotein 120 (gp120) and protein 27 (p27) alone; (●) gp120 + p27 + granulocyte–macrophage colony-stimulating factor (GM-CSF) + interleukin (IL)-2; (□) gp120 + p27 + GM-CSF + IL-4. The proportion of $CCR5^+$ cells was determined by flow cytometry, β-chemokines by enzyme-linked immunosorbent assay (ELISA) and plasma SIVmac RNA by the SIVmac bDNA assay (Chiron Diagnostics). Polymerase chain reaction analyses were also carried out and these were positive in all but one macaque, in which SIVmac RNA was not detectable in the plasma. SIV co-culture was significantly correlated with the plasma SIVmac RNA results ($r = 0{\cdot}71$, d.f. 7, $P < 0{\cdot}05$).

We suggest a dual objective in immunization against HIV or SIV: to elicit both cognate (specific) immunity and innate or non-specific up-regulation of β-chemokines. The vaccine and route of immunization can be designed to elicit cellular and humoral immunity against HIV and to up-regulate the β-chemokines that bind and down-modulate the co-receptors, exerting a complementary effect in preventing or decreasing HIV infection. This dual strategy is aimed at the three SIV or HIV barriers: the mucosal site of entry, the draining lymph nodes and circulating blood, as reviewed recently.[33]

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CONCISE COMMUNICATION

Antigen-Specific Production of RANTES, Macrophage Inflammatory Protein (MIP)-1α, and MIP-1β In Vitro Is a Correlate of Reduced Human Immunodeficiency Virus Burden In Vivo

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RANTES (regulated on activation, normal T expressed and secreted), macrophage inflammatory protein (MIP)–1α, and MIP-1β are human immunodeficiency virus (HIV) suppressor factors by virtue of their ability to compete with HIV for access to cell surface R5. Their ability to block HIV infection in vitro is unequivocal; however, their role as HIV suppressor factors in vivo is not firmly established. We therefore conducted a study to test the hypothesis that production of these factors in vitro was a correlate of decreased virus burden in vivo. Moreover, we asked whether higher β chemokine production could be demonstrated with cells from people who are R5D32 heterozygotes, compared with people who are R5 wild-type homozygotes. Our data support the thesis that RANTES, MIP-1α, and MIP-1β production is associated with decreased in vivo virus load. Moreover, enhanced production of these factors may be explained in part by the genetic background of the host.

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All subjects provided informed consent, and guidelines of the US Department of Health and Human Services were followed in the conduct of this research.

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Infection without disease progression for ≥10 years occurs in a minority of human immunodeficiency virus (HIV)–infected people. Host factors, including chemokine production [1, 2] and chemokine receptor polymorphisms (reviewed in Roger [3]), may contribute to this finding. The goal of this study was to evaluate whether HIV antigen–stimulated production of 3 natural R5 ligands (RANTES [regulated on activation, normal T expressed and secreted], macrophage inflammatory protein [MIP]–1 α, and MIP-1β) and memory HIV cytotoxic T lymphocyte (CTL) activity were associated with decreased virus load in vivo. Whether chemokine production or memory CTL activity was related to R5D32 heterozygosity was also tested. These relationships have been difficult to establish and they remain a point of controversy [4].

The present investigation was conducted with antiretroviral drug–naive people, to accurately assess these host-virus relationships, without the confounding effect of therapy. In addition, long-term survivors were studied exclusively, to control for the duration of HIV infection. Our assay targeted antigen-specific $CD8^+$ cell responses by use of HIV-recombinant vaccinia viruses, to stimulate chemokine production in tandem with expansion of memory CTL responses.

The data provide evidence for a significant inverse correlation between antigen-stimulated augmented RANTES, MIP-1α, and MIP-1β production and reduced in vivo virus burden. In addition, augmented β-chemokine production was found in people classified as R5D32 heterozygotes, compared with R5 wild-type donors. In contrast, the memory CTL response was not found to be significantly associated with in vivo HIV burden, irrespective of whether the data set was analyzed as a whole or stratified according to host R5 genotype.

Materials and Methods

Study volunteers. The entry criteria for this study was HIV infection with long-term survival, which was defined as >10 years' survival, with documented infection, and no reported use of antiretroviral agents. A total of 55 such people were identified from the Los Angeles site of the Multicenter AIDS Cohort Study and were solicited for their participation. Thirty-two of 55 people agreed to participate and remained antiretroviral drug naive; therefore, they were the focus of this investigation.

$CD8^+$ cell stimulation and measures of effector function. $CD8^+$ cells were purified to >90% by negative immunomagnetic selection (magnetic cell sorter, Miltenyi Biotech, Bergish Gladbach, Germany). We stimulated 3×10^6 $CD8^+$ cells per well with one-tenth as many autologous peripheral blood mononuclear cells that were infected with HIV-recombinant vaccinia expression vectors [5]. Cell-free culture supernatants were harvested after 24 h of stimulation and were tested for RANTES, MIP-1α, and MIP-1β levels by ELISA (R&D Systems, Minneapolis). For the purpose of data analysis, the levels

of RANTES, MIP-1β, and MIP-1β were added together and expressed as a single analyte, because all 3 proteins effectively suppress HIV replication. According to the convention of the memory CTL assay, levels of RANTES, MIP-1α, and MIP-1β produced by stimulation with wild-type vaccinia virus were subtracted from the levels produced in response to stimulation by the HIV-recombinant vaccinia vector. Although measurable RANTES, MIP-1α, and MIP-1β production resulted from stimulation by wild-type vaccinia virus, these levels were always lower relative to stimulation with the HIV-recombinant vaccinia constructs. Moreover, the statistical significance of the results was unchanged when the data (table 1) were analyzed without subtracting RANTES, MIP-1α, and MIP-1β levels produced in the vaccinia control cultures (data not shown).

Table 1. Comparison of plasma virus load versus CTL, β chemokine production, and CD38 expression on $CD8^+$ cells and $CD4^+$ cell levels in 32 antiretroviral drug–naive people.

Memory CTL assays were performed on cells expanded after 7 days of stimulation. Targets were autologous Epstein-Barr virus–immortalized B cells infected with HIV-recombinant vaccinia expression vectors, as described elsewhere [5]. These data were expressed as lytic units.

Flow cytometry. Levels of $CD4^+$ cells were derived by flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA), and a differential count was derived by standard methods. CD38 expression on $CD8^+$ cells was derived by multiplying the channel number corresponding to the median relative fluorescence intensity of phycoerythrin (PE)–CD38 staining on $CD8^+$ cells by the number of PE molecules detected per channel [6].

HIV RNA polymerase chain reaction. Plasma RNA levels were measured by the Roche HIV-1 Amplicor kit (Roche Molecular Systems, Branchburg, NJ) according to the manufacturer's instructions. R5 genotype was performed on genomic DNA with DNAzol according to the manufacturer's instructions (GIBCO BRL, Rockville, MD). R5 primer and amplification conditions were as described elsewhere [7].

Results

These data show a significant inverse correlation between plasma HIV RNA in vivo and production of RANTES, MIP-1α, and MIP-1β in vitro (table 1; $P = .002$). Chemokine production varied according to the donors' plasma virus burden ($n = 8$ donors per quartile) and ranged from a median of 1200 pg/mL in those with the lowest virus burden (range, <200–519 HIV copies/mL) to 200 pg/mL in those with the highest virus burden (range, 30,129–442,266 HIV copies/mL). Purified $CD8^+$ cells from control subjects uninfected with HIV ($n = 5$) showed minimal levels of antigen-specific RANTES, MIP-1α, and MIP-1β production (median, 0 pg/mL; range, 0–200 pg/mL; data not shown). As a positive control, we measured the CD38 activation marker on $CD8^+$ cells. CD38 expression patterns on $CD8^+$ cells are accurate flow cytometric representations of the $CD8^+$ cell response to HIV that not only reflect disease pathogenesis [5] but also carry prognostic value [8]. As described elsewhere [8], we found that this measure of $CD8^+$ cell activation was positively correlated to plasma HIV RNA levels (table 1; $P = .003$).

In contrast to these relationships was the finding that Gag-specific $CD8^+$ CTL responses did not differ in magnitude across the quartiles (table 1; *P*, not significant [NS]). Other reports that CTLs are negatively correlated with virus burden (reviewed in Autran et al. [9]) were not substantiated in this study. This is possibly due to methodological differences between our study and others, inasmuch as we did not calculate the precursor frequency per se, but rather cultivated the $CD8^+$ CTLs in bulk cultures.

Our data were also stratified according to the R5 genotype of each person, to test the hypothesis that reduced cell-surface expression of R5 in people classified as R5D32 heterozygotes would be associated with higher $CD4^+$ cell counts and higher RANTES, MIP-1α, and MIP-1β production. Overall, the frequency of R5D32 heterozygosity (7 of 32; 22%) that we observed was consistent with that reported large-scale epidemiological studies reviewed in Roger [3]. Our analysis demonstrated that cells from R5D32 heterozygotes produced nearly 4-fold more RANTES, MIP-1α, and MIP-1β, compared with R5 homozygous wild-type donors (table 2; $P = .009$). The observed association between R5D32 heterozygosity and enhanced RANTES, MIP-1α, and MIP-1β production has been reported elsewhere in the setting of exposed but uninfected people [10] and is fully consistent with the relationships in this study. Similar to observations in table 1, memory $CD8^+$ CTL activity was present at a similar magnitude in both groups and was, therefore, independent of R5 genotype (table 2; *P* = NS).

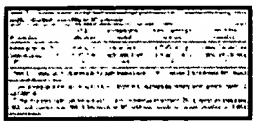

Table 2. Comparison of viral and immunologic parameters in 32 antiretroviral drug–naive people, stratified according to R5 genotype.

Finally, our results show that R5D32 heterozygosity is associated with higher CD4 counts and reduced virus load (table 2) in this group of people who had been infected for ≥10 years. Statistical significance, however, was demonstrated for $CD4^+$ cell counts only ($P = .03$). The lack of statistical significance for HIV plasma RNA levels may reflect small sample size. Alternatively, R5D32 heterozygosity could potentially limit $CD4^+$ T cell destruction by mechanisms that are independent of virus burden.

Discussion

The findings of this study add weight to the accumulating evidence [1, 2] that augmented production of RANTES, MIP-1α, and MIP-1β may limit viral replication in vivo, thereby reducing destruction of host $CD4^+$ T cells. In this regard, our results do not preclude the possibility that other cytokines or chemokines, including $CD8^+$ cell antiviral factor [4], could also play an important role. On another front, these data extend previous accounts that the genetic background of the host may be positively associated with the functional capacity of the immune system to mount an effective immune defense against HIV. Our results are the first to indicate that heterozygous R5D32 donors produce enhanced levels of β chemokines in response to antigen-specific stimulation.

Additional studies are underway in our laboratory to further clarify the cellular mechanisms that underlie the enhanced production of RANTES, MIP-1α, and MIP-1β that was observed. The first

addresses whether $CD8^+$ cells are indeed the primary source of the β chemokines in our cultures. This possibility is supported by our experimental design, which was based on methods used to activate and expand antigen-specific memory $CD8^+$ CTLs in vitro; >90% of the cells in our cultures were $CD8^+$ cells. Knowledge that β chemokines are stored within intracytoplasmic granules of $CD8^+$ cells for rapid release [11] and that our measurements were made from 24-h supernatants provides further support for this hypothesis, decreasing the likelihood that $CD4^+$ cells [1], monocytes [12], or NK cells [13] (all present in low frequencies in our cultures) were major contributors to the RANTES, MIP-1α, and MIP-1β levels. Direct evidence will require a detailed analysis of β chemokine production at the single-cell level, and such assays are currently under development in our laboratory.

Chemokines are metabolized in concert with their production [14], and, in most cases, the rate of metabolism is assumed to be invariant among donor cells. It is known, however, that R5—which binds and internalizes RANTES, MIP-1α, and MIP-1β—can be expressed at different levels at the cell surface according to cell maturation state [15] or host genotype [7]. Thus, it is possible that the levels of secreted β chemokines that were observed in culture supernatants from our donors partially reflect ligand receptor density on the cell surface. This hypothesis is supported by the data presented in table 2, which indicates that culture supernatants derived from R5D32-heterozygous people had nearly 4-fold higher levels of RANTES, MIP-1α, and MIP-1β relative to R5 homozygous wild-type people. In effect, fewer R5 receptors per cell could augment extracellular chemokine levels through decreased receptor-mediated uptake.

In summary, our results show that the levels of RANTES, MIP-1α, and MIP-1β produced in response to stimulation with HIV proteins was significantly associated with reduced viral replication in vivo. RANTES, MIP-1α, and MIP-1β production in vitro, therefore, appears to be a correlate of immunity in the HIV model. The results of this study suggest that HIV disease nonprogression in the setting of R5 expression could operate in combination with enhanced production or reduced metabolism of RANTES, MIP-1α, and MIP-1β. These findings may have important implications with respect to vaccine design and development of novel therapeutics, because this study adds strength to the argument that host factors responsible for resistance to infection or delayed disease progression may be related to host genotype, as well as to antigen-specific production of RANTES, MIP-1α, and MIP-1β—and perhaps additional HIV suppressor factors—by $CD8^+$ cells.

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Differential Regulation of CC Chemokine Gene Expression in Human Immunodeficiency Virus-Infected Myeloid Cells

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The importance of chemokine expression on HIV infection has been emphasized by the discovery that infection of $CD4^+$ T cells by M-tropic strains of HIV-1 is antagonized by the chemokines RANTES, MIP-1α, and MIP-1β, which are natural ligands of CCR5, a major coreceptor for macrophagetropic (M-tropic) isolates of HIV-1. Similarly, the CCR2b ligands MCP-1 and MCP-3 inhibit productive infection of PBMCs by both CCR5- and CXCR4-dependent strains of HIV-1, suggesting that expression of the MCP-1 chemokine may affect HIV infection via signaling through the CCR2 receptor and subsequent desensitization of the CCR5 and/or CXCR4 signaling pathway. Given the major role played by chemokine receptors in HIV-1 fusion/entry and the regulatory effects of chemokines on HIV-1 infection, we examined the pattern of chemokine gene expression in HIV-1-infected myeloid cells and in primary monocyte/macrophages. Chronic HIV-1 infection of U937 monocytic cells increased the expression of RANTES, MIP-1α, MIP-1β, and IL-8 chemokine genes, but strongly inhibited PMA/PHA- and TNFα-induced MCP-1 gene transcription. HIV-1-mediated inhibition of MCP-1 transcription and secretion was further confirmed in *de novo* HIV-1-infected U937 cells and correlated with a delay in HIV- and signal-induced NF-κB binding to the MCP-1 promoter. The inhibition of MCP-1 gene expression may provide a mechanism by which HIV-1 escapes the early influence of chemokine expression in monocytic cells.

INTRODUCTION

Human immunodeficiency virus (HIV) infects a number of cell types, including $CD4^+$ T cells (Gallo *et al.*, 1984), monocytes (Gartner *et al.*, 1986), and macrophages (Koenig *et al.*, 1986), as well as bone marrow precursor cells (Stanley *et al.*, 1992), both *in vivo* and *in vitro*. In contrast to HIV replication in T lymphocytes, which results in T cell death, infection of cells of the monocytic lineage produces high levels of virus in the absence of significant cytopathicity (Roy and Wainberg, 1988; McElrath *et al.*, 1989), suggesting that infected monocytes/macrophages (M/M) may serve as a reservoir for HIV persistence and spread *in vivo* (Meltzer *et al.*, 1990; Meltzer and Gendelman, 1992; Perno *et al.*, 1997). Promonocytic cell lines such as U937 and THP-1 have been widely used as *in vitro* models to investigate HIV-1 infection of M/M, although these cells differ markedly from their *in vivo* counterparts in terms of their susceptibility to different viral strains (Schuitemaker *et al.*, 1992a; b). U937 cells become susceptible to macrophage-tropic (M-tropic) HIV-1 after treatment with retinoic acid (RA) or phorbol 12-myristate 13-acetate (PMA), agents that induce myeloid differentiation and expression of CCR5, a major fusion/entry cofactor for M-tropic HIV-1 (Moriuchi *et al.*, 1998).

HIV infection is initiated by viral envelope glycoprotein gp120 interaction with cell surface CD4, followed by association with a coreceptor that triggers fusion of viral and cellular membranes (Deng *et al.*, 1996; Feng *et al.*, 1996; Doms and Peiper, 1997). Most monocytotropic HIV-1 strains use CCR5 chemokine receptor to enter macrophages and primary T lymphocytes (Dragic *et al.*, 1996; Raport *et al.*, 1996; Alkhatib *et al.*, 1996), whereas T-tropic HIV-1 strains use CXCR4 (fusin, LESTR, or HUMSTR) for primary $CD4^+$ T lymphocytes and $CD4^+$ T cell lines (Feng *et al.*, 1996; Berson *et al.*, 1996). In addition, other chemokine receptors support infection by one or more virus strains *in vitro*, including CCR2b and CCR3 (Doranz *et al.*, 1996; Choe *et al.*, 1996; Rucker *et al.*, 1996). The predominant virus strains isolated early after infection from asymptomatic, HIV-positive individuals use CCR5 as coreceptor, while viruses that emerge later during the course of infection use CXCR4 either in place of or in addition to CCR5 (Berger *et al.*, 1998).

While coreceptors play a critical role in supporting entry of HIV-1 into cells, coreceptors may also influence postentry events (Edinger *et al.*, 1997; Chackerian *et al.*, 1997). Interaction of soluble HIV-1 and SIV Env proteins with CCR5 and CXCR4 results in receptor signaling (Davis *et al.*, 1997; Weissman *et al.*, 1997). Coreceptor

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signaling (coupling, phosphorylation, and internalization) are not required for Env-mediated membrane fusion or virus infection of transformed cell lines, but receptor signaling mediated either by HIV-1 interaction or by binding of ligand could influence postentry events of virus replication in primary cells such as macrophages (Aramori *et al.*, 1997).

Chemokines are small (8- to 12-kDa) chemoattractant proteins that constitute the natural ligands for chemokine receptors. The β-chemokines RANTES, MIP-1α, and MIP-1β interact with CCR5 (Raport *et al.*, 1996) and the α-chemokine stromal-derived factor-1 (SDF-1) serves as a ligand for CXCR4 (Bleul *et al.*, 1996). Consequently, these chemokines can block infection of HIV-1 strains that use CCR5 and CXCR4, respectively. For example, RANTES, MIP-1α, and MIP-1β inhibit infection of CCR5-expressing peripheral blood mononuclear cells (PBMCs) by macrophage-tropic virus, while SDF-1 blocks infection of $CXCR4^+$ HeLa/CD4 cells by the T cell line-adapted HIV-1 LAI (Cocchi *et al.*, 1995; Bleul *et al.*, 1996; Oberlin *et al.*, 1996). Given these inhibitory effects, increased chemokine production may function as part of a protective host immune response against HIV infection and disease progression (Cocchi *et al.*, 1995). Thus, β-chemokine gene expression is strongly enhanced in lymph nodes of patients with HIV disease, indicating that cells recruited to HIV-infected lymph nodes are likely to interact with β-chemokines before exposure to virus, since these molecules direct mononuclear cell traffic to sites of inflammation (Tedla *et al.*, 1996; Adams and Lloyd, 1997). However, conflicting results have reported different effects of β-chemokine expression on HIV-1 replication in MDM and tissue macrophages (Dragic *et al.*, 1996; Simmons *et al.*, 1997; Schmidtmayerova *et al.*, 1996; Coffey *et al.*, 1997).

Given the major role played by chemokine receptors in HIV-1 fusion/entry and the regulatory effects of chemokines on HIV-1 infection, we sought to examine the pattern of chemokine gene expression in HIV-1-infected monocytic U937 cells and primary monocyte-derived macrophages (MDM). In uninfected cells, PMA/PHA treatment induced expression of β-chemokines such as RANTES, MCP-1, and MIP-1α and β, as well as the α-chemokines IL-8 and γ-IP10. Chronic and *de novo* HIV-1 infection of U937 cells increased the expression of these chemokine genes, but strongly inhibited PMA/PHA- and TNFα-induced MCP-1 gene transcript levels and MCP-1 protein secretion. HIV infection of U937 resulted in a delayed induction of NF-κB binding to the MCP-1 promoter and correlated with the repression of PMA/PHA- and TNFα-induced MCP-1 transcription. The inhibition of MCP-1 gene expression suggests a mechanism by which HIV-1 may escape the early influence of chemokine expression in monocytic cells.

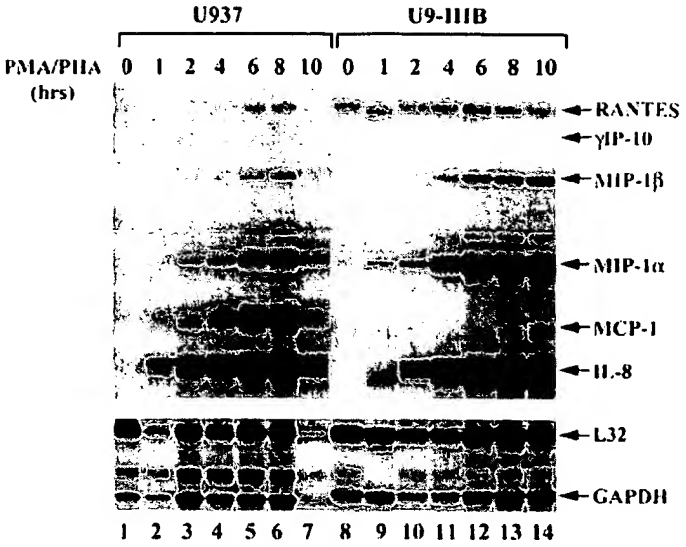

FIG. 1. Kinetics of chemokine induction in PMA/PHA-stimulated U937 and U9-IIIB cells. U937 promonocytic and chronically HIV-1-infected U9-IIIB cells were left unstimulated (lanes 1 and 8) or treated with PMA (50 ng/ml) and PHA (10 ng/ml) for 1, 2, 4, 6, 8, and 10 h (lanes 2–7 and 9–14). Total RNA extracted (5 μg) from these cells was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit (PharMingen, San Diego, CA). Arrows indicate the migrations of labeled fragments protected from RNase digestion and corresponding either to chemokine mRNA (RANTES, γ-IP10, MIP-1β, MIP-1α, MCP-1, and IL-8) or to a control gene mRNA (L32 and GAPDH).

RESULTS

Differential regulation of chemokine gene expression in chronically infected monocytic cell lines

The aim of this study was to examine the effect of the HIV infection on chemokine gene expression in myeloid cell lines (U937), monocytes, and MDM. To determine the pattern of chemokine expression in monocytic cell lines, promonocytic U937 cells were stimulated with PMA/PHA and harvested at different times after stimulation. RNA extracted from these cells was then subjected to RNase protection analysis. Expression levels of the α-chemokines IL-8 and γ-IP10, the β-chemokines RANTES, MIP-1α, MIP-1β, MCP-1, and I-309, and the γ-chemokine lymphotactin (Rollins, 1997; Baggiolini *et al.*, 1997) were determined and quantified by normalization with L32 and GAPDH mRNA levels as internal controls.

In the absence of stimulation, low-level expression of chemokine RNA was detected in U937 cells (Fig. 1, lane 1). PMA/PHA treatment of U937 cells lead to a dramatic increase (>200-fold after 8 h of stimulation) in the expression of MIP-1α, MCP-1, and IL-8, as well as a modest increase (2- to 10-fold) in RANTES, γ-IP-10, and MIP-1β mRNA levels (Fig. 1, lanes 2–7), in agreement with the induced expression of MCP-1, MIP-1α, MIP-1β, and IL-8 as observed previously (Biswas *et al.*, 1998). Induction appeared to be coordinately regulated and reached a maximum at 8 h after stimulation (Fig. 1, lane 6). As

expected, no mRNA corresponding to T-cell-restricted lymphotactin or I-309 genes was detected in U937 cells (data not shown).

The effect of HIV-1 infection on uninduced or PMA/PHA-induced expression of chemokine genes was examined initially using U9-IIIB cells chronically infected with the HIV-1 IIIB strain (Fig. 1, lanes 8–14). In uninduced U9-IIIB cells, RANTES and MIP-1α mRNA were upregulated compared to noninfected U937 cells (Fig. 1, compare lanes 1 and 8). Stimulation of U9-IIIB cells with PMA/PHA increased MIP-1α, MIP-1β, and IL-8 mRNA expression with the same kinetics as observed in U937 cells (Fig. 1, lanes 9–14); the induced levels of chemokine RNA were increased 3- to 4-fold in infected cells compared to uninfected cells, indicating that HIV infection enhanced chemokine gene expression. Surprisingly, in HIV-infected U9-IIIB cells, MCP-1 mRNA levels were reduced dramatically; after 8 h of PMA/PHA stimulation, a 15-fold decrease in MCP-1 mRNA was detected (Fig. 1, lanes 9–14). Similarly, only low-level expression of γ-IP10 was observed after PMA/PHA induction in chronically infected U9-IIIB cells.

Regulation of chemokine expression during *de novo* HIV infection of U937 cells

De novo infection of U937 cells was performed to determine the pattern of chemokine expression during the course of HIV-1 infection; the progress of infection was monitored by reverse transcriptase activity (Fig. 2A). At different times after infection, cells were treated with either PMA/PHA or TNFα for 8 h and then analyzed for chemokine RNA accumulation. *De novo* HIV infection of U937 cells differentially modulated chemokine RNA expression (Fig. 2B); in unstimulated cells, the level of MCP-1 RNA decreased during the course of infection, whereas constitutive RANTES expression was not affected by infection (Fig. 2B, lanes 1–5). PMA/PHA-induced MCP-1 transcription was inhibited significantly after 6 and 8 days of infection (Fig. 2B, compare lanes 6, 8, and 9). Residual mRNA levels could be due to MCP-1 expression in uninfected cells. MCP-1 transcript levels were restored by day 24; the apparent decrease was due to lower recovery of RNA from 24 day-infected cells, as indicated by the weak signals for L32 and GAPDH (Fig. 2B, lane 10). Quantification of MCP-1 mRNA levels, using L32 and GAPDH as internal controls, revealed a higher MCP-1/L32 mRNA ratio at day 24 than at day 8 (see Fig. 3A). In contrast to chronic infection, *de novo* infection of U937 cells resulted in a significant decrease in PMA/PHA-induced MIP-1α, MIP-1β, and RANTES mRNA (Fig. 2B, lanes 6–8). This downregulation presented the same transient characteristics as observed for the MCP-1 gene (Fig. 2B, compare lane 6 and lanes 7–10). A similar decrease in MCP-1 mRNA levels was obtained

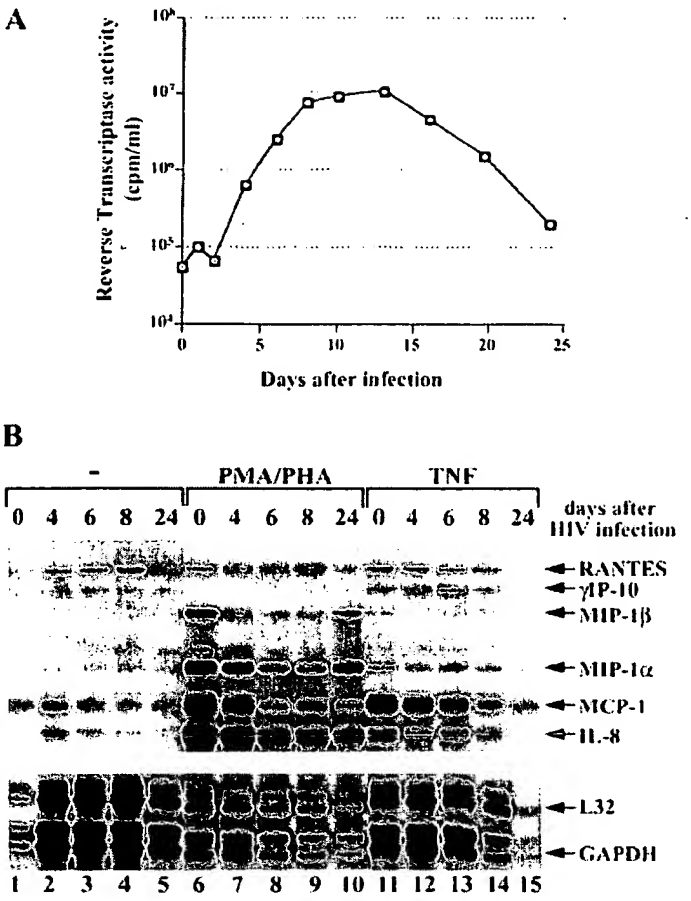

FIG. 2. Regulation of chemokine expression during *de novo* HIV infection of U937 cells. U937 myelomonoblastic cells were infected for 2 h with the HIV-1 strain IIIB at an m.o.i. of 0.3 PFU/ml. (A) At different days after infection, the efficiency of infection was monitored by determination of reverse transcriptase activity in the supernatants of infected cells. Supernatant (50 μl) was centrifuged for 30 min at 3000 rpm (4°C), mixed with reverse transcriptase reaction buffer, and incubated 22 h at 30°C to measure [^{3}H]TTP incorporation into acid-precipitable nucleic acid. The reverse transcriptase activity (cpm/ml) was normalized according to cell concentration and plotted in a log scale. (B) At days 4 (lanes 2, 7, and 12), 6 (lanes 3, 8, and 13), 8 (lanes 4, 9, and 14), and 24 (lanes 5, 10, and 15) after infection, 10 $\times$ 10^6 cells were left unstimulated (lanes 1–5) or treated either with PMA (50 ng/ml) and PHA (10 ng/ml) (lanes 6–10) or with TNFα (10 ng/ml, lanes 11–15) for 8 h. Total RNA (5 μg) extracted from these cells was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit (PharMingen). For PMA/PHA-stimulated conditions (lanes 6–10), a 4-h exposure of the gel is presented, whereas a 16-h exposure of the gel is shown for the unstimulated (lanes 1 to 5) and TNFα-stimulated conditions (lanes 11 to 15).

when HIV-infected cells were stimulated with TNF-α, although mRNA of other chemokines were barely detectable after 8 h of TNF-α stimulation (Fig. 2B, lanes 11–15). To confirm that this downregulation was solely due to HIV infection, U937 cells were cultured for 4, 6, and 8 days and stimulated or not with PMA/PHA or TNFα for 8 h. No differences in the constitutive or induced chemokine expression pattern were observed during this period, indicating that the downregulation shown at early time of infection was specific to HIV-1 (data not shown).

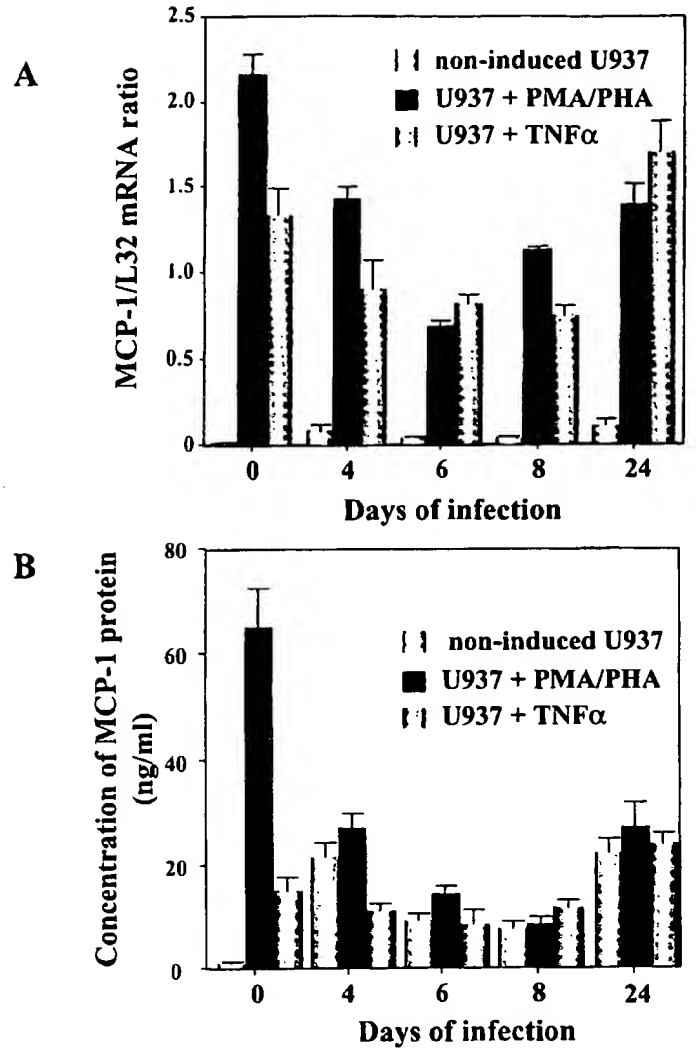

FIG. 3. Downregulation of MCP-1 mRNA and protein levels by *de novo* HIV infection of U937 cells. U937 myelomonoblastic cells were infected for 2 h with the HIV-1 strain IIIB. (A) Quantification of the RNase protection assay shown in Fig. 2B. For each lane, the labeled fragment protected from RNase digestion and corresponding to MCP-1 mRNA was quantified (graph at the top of the figure), using a Scan Jet 4c Hewlett–Packard scanner and NIH Image 1.60 software. The determined values were normalized according to the L32 mRNA levels and plotted as MCP-1/L32 mRNA ratios. Similar results were obtained in three independent experiments when the MCP-1 mRNA signal was normalized according to the GAPDH mRNA. (B) The concentration of secreted MCP-1 protein was determined from the supernatants of cultures (50 μl) of infected U937 treated with PMA/PHA or TNFα, using the human monocyte chemoattractant protein-1 (hMCP-1) ELISA kit (BioSource Int., Camarillo, U.S.A.).

Downregulation of MCP-1 mRNA and protein levels in *de novo* infected U937 cells

Quantification of MCP-1 mRNA levels during HIV infection demonstrated that PMA/PHA-induced transcription of MCP-1 was decreased more than threefold after 6 days of infection compared to levels in infected cells (Fig. 3A). Furthermore, uninduced and TNF-α-induced levels of MCP-1 RNA were also subject to the same downregulation, although the effect was less marked (Fig. 3A). To assess whether the amount of MCP-1 protein was similarly altered in HIV-infected U937 cells, secreted MCP-1 was measured by ELISA in supernatants of HIV-1-infected cells. Concomitant with the decrease in MCP-1 mRNA, the amount of MCP-1 secreted by PMA/PHA-induced cells decreased throughout HIV infection; by day 8, MCP-1 levels were reduced sixfold compared to uninfected cells (Fig. 3B). Interestingly, the decreased MCP-1 levels were nearly identical to those measured in untreated 8 day-infected cells, indicating that HIV infection completely abolished the capacity of U937 cells to secrete MCP-1 in response to PMA/PHA stimulation. Secretion of MCP-1 by uninfected and TNF-α-treated cells was also inhibited by HIV infection until day 8 (Fig. 3B). By 24 days of infection, the levels of MCP-1 protein were partially restored, as seen at the mRNA level (Figs. 3A and 3B). Quantification of RANTES mRNA levels and measurement of secreted RANTES showed a modest decrease (around 50%) of mRNA and a dramatic decrease of RANTES secretion in early times—days 4 and 6—of HIV infection in PMA/PHA- and TNFα-stimulated cells (data not shown).

MCP-1 transcription is downregulated after *in vitro* differentiation of primary monocytes

Primary monocytes/macrophages were isolated from peripheral blood of uninfected individuals and examined for PMA/PHA-induced chemokine expression. The expression pattern in primary monocytes was almost identical to that observed in U937 cells (Fig. 4A, lanes 3 and 5). These data indicated that U937 cells reflected the characteristics of *in vivo* primary monocytes, at least in terms of the regulation of chemokine expression. This observation was confirmed by the marked differences between primary monocytes and nonadherent lymphoid cells (Fig. 4A, compare lanes 2 and 5). In contrast to unstimulated monocytes, which only weakly expressed chemokine RNA in the absence of stimulation (Fig. 4A, lane 4), constitutive transcription of RANTES, γIP-10, MIP-1α and β, MCP-1, and IL-8 was observed in lymphoid cells (Fig. 4A, lane 1). Furthermore, significant expression of lymphotactin—a T-cell-specific chemokine—was detected; also PMA/PHA stimulation of lymphoid cells slightly enhanced the mRNA levels of MIP-1α and β, but reduced that of MCP-1 (Fig. 4A, compare lanes 1 and 2).

In vitro culture of primary monocytes resulted in the downregulation of PMA/PHA-induced MCP-1 mRNA levels at 3, 6, and 9 days after seeding, but did not significantly affect expression of MIP-1α, MIP1-β, and IL-8 genes (Fig. 4, lanes 6 and 7). Interestingly, a significant downregulation of RANTES expression was also observed in primary monocytes after 3, 6, and 9 days of *in vitro* differentiation (Fig. 4, lanes 4–7). These data indicated that expression of MCP-1 RNA were downregulated when primary monocytes were differentiated into

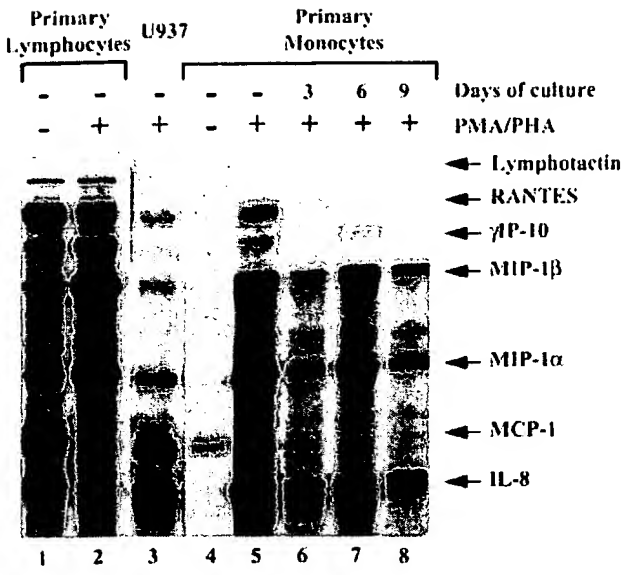

FIG. 4. *In vitro* differentiation of primary monocytes extracted from blood. Primary monocytic cells were purified by the adherence separation method from PBMCs—extracted from blood by Ficoll separation. Cells from the adherent fraction were cultured for 24 h (lanes 4 and 5) or 3, 6, or 9 days (lanes 6–8). At days 0 (lane 5), 3 (lane 6), 6 (lane 7), or 9 (lane 8), cells were treated with PMA (50 ng/ml) and PHA (10 ng/ml) for 8 h. PMA/PHA-stimulated U937 (lane 3) and unstimulated or PMA/PHA-treated lymphoid cells from the nonadherent fraction (lanes 1 and 2) were used as controls. Total RNA (5 μg) extracted from these cells was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit (PharMingen).

macrophages. A similar observation was made when U937 cells were infected by the HIV-1 IIIB strain for the same period of time (data not shown). The similarities in MCP-1 and RANTES inhibition observed after HIV infection or after monocytic differentiation suggest a common mechanism resulting in MCP-1- and RANTES-specific repression.

Effect of antioxidant agents on chemokine expression in monocytic lineages

Chronic infection of myeloid U937 and PLB985 cells leads to constitutive NF-κB activity, due to enhanced IκBα turnover and increased NF-κB induction (Roulston *et al.*, 1992; 1993; DeLuca *et al.*, 1996). To assess the potential role of NF-κB in the coordinated regulation of chemokine genes, cells were treated with *N*-acetyl-L-cysteine (NAC) and pyrrolidinedithiocarbamate (PDTC)—antioxidant agents that specifically inhibit the NF-κB induction in a variety of cell types (DeLuca *et al.*, 1998; Lee *et al.*, 1997). NAC and PDTC treatment of U937 cells inhibited PMA/PHA-induced transcription of RANTES, MIP-1α, MIP-1β, and MCP-1 and reduced IL-8 gene expression (Fig. 5, lanes 4–6 of the corresponding panels). However, PMA/PHA-induced expression of MIP-1α and IL-8 was less sensitive to NAC or PDTC treatment in U9-IIIB cells than in uninfected U937 cells (Fig. 5, compare lanes 4–6 to 10–12 in each panel). Since NAC and PDTC have been shown to inhibit the induction of NF-κB by PMA, the upregulation of MIP-1α- and IL-8-induced transcription in chronically infected U9-IIIB cells cannot be explained by sustained NF-κB activity in these cells. As expected, no effect of NAC or PDTC treatment was observed on MCP-1 and γ-IP10 expression in U9-IIIB cells. Similar results were obtained after stimulation of uninfected and HIV-infected cells by TNFα (data not shown). The inhibition of PMA/PHA-induced transcription of MCP-1 gene expression in U937 cells by NAC and PDTC treatment strongly suggests that activation of NF-κB activity is responsible for induced expression of these two genes and that chronic or *de novo* HIV infection may lead to decreased or inhibited NF-κB binding activity in U937 cells.

NF-κB binding to the A1-κB site of the MCP-1 promoter is inhibited by HIV

The MCP-1 promoter contains in its distal region two NF-κB sites—A1 and A2, located at −2645 to −2626 and

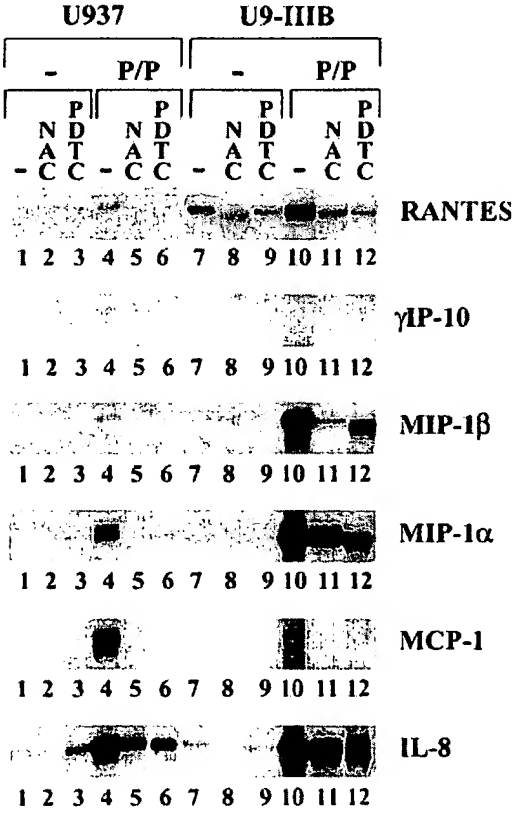

FIG. 5. Effect of antioxidant agents on chemokine expression in unstimulated or PMA/PHA-stimulated U937 and U9-IIIB cells. U937 promonocytic and chronically HIV-1-infected U9-IIIB cells were untreated (lanes 1, 4, 7, and 10) or pretreated with *N*-acetylcysteine (NAC, lanes 2, 5, 8, and 11) or pyrrolidinedithiocarbamate (PDTC, lanes 3, 6, 9, and 12) for 1 h. Cells were then left unstimulated (lanes 1–3 and 7–9) or treated with PMA (50 ng/ml) and PHA (10 ng/ml) for 8 h (lanes 4–6 and 10–12) in the continuous presence of antioxidant agents. Total RNA (5 μg) extracted from these cells was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit (PharMingen).

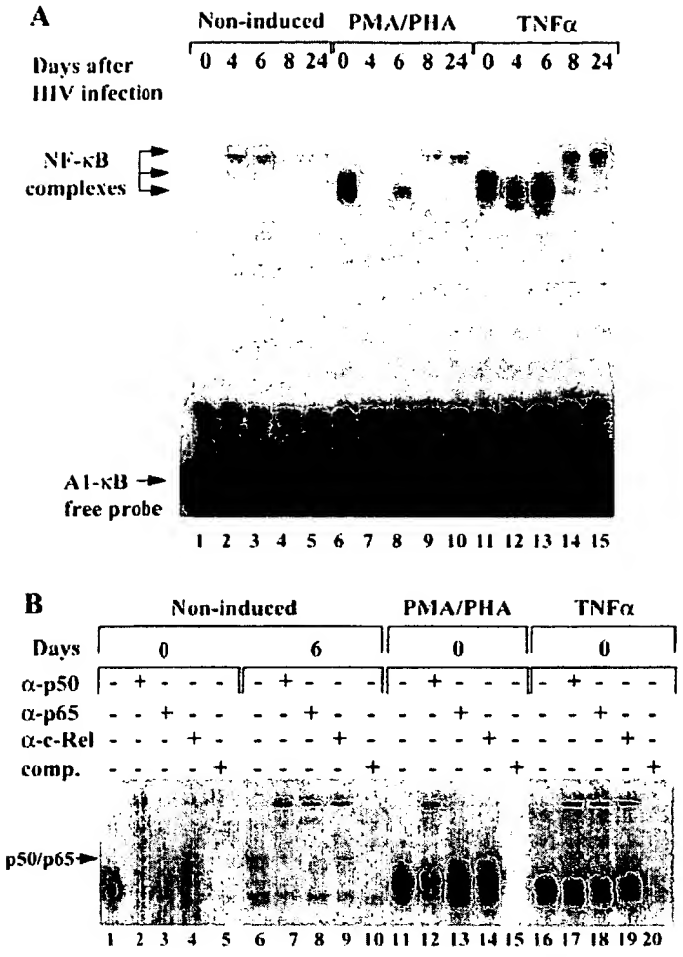

FIG. 6. Inhibition of induced NF-κB binding activity to the A1-κB site of the MCP-1 promoter. U937 myelomonoblastic cells were infected for 2 h with the HIV-1 strain IIIB. (A) Before infection (lanes 1, 6, and 11) or at days 4 (lanes 2, 7, and 12), 6 (lanes 3, 8, and 13), 8 (lanes 4, 9, and 14), and 24 (lanes 5, 10, and 15) after infection, 5×10^6 cells were left unstimulated (lanes 1–5) or treated either with PMA (50 ng/ml) and PHA (10 ng/ml) (lanes 6–10) or with TNFα (10 ng/ml, lanes 11–15) for 8 h. Whole cell extracts (10 μg) were subjected to EMSA analysis using [γ-^{32}P]ATP-labeled A1-κB probe. Arrows indicate the position of specific NF-κB complexes. (B) Supershift analysis was performed with extracts from uninfected (lanes 1–5 and 11–20) U937 cells or cells infected by HIV-1 for 6 days (lanes 6–10) using anti-p50 (lanes 2, 7, 12, and 17), anti-p65 (lanes 3, 8, 13, and 18), or anti-c-Rel (lanes 4, 9, 14, and 19) antibodies with A1-κB probe. Competition was performed in the presence of a 100-fold excess of unlabeled A1-κB oligonucleotide (lanes 5, 10, 15, and 20). Arrow indicates the position of complex shifted by both anti-p50 and anti-p65 antibodies.

−2616 to −2597, respectively—which are involved in TPA-induced expression of MCP-1 (Ueda *et al.*, 1997). NF-κB binding to the MCP-1 promoter was assessed in HIV-infected U937 cells by EMSA using the A1-κB site of the MCP-1 promoter as probe. In control U937 extracts, two weak NF-κB complexes were detected which reacted with anti-p50 antibody (Figs. 6A, lane 1, and 6B, lanes 1–5). Stimulation of U937 cells with PMA/PHA or TNFα resulted in an approximately 10-fold induction of the two complexes (Fig. 6A, lanes 1, 6, and 11). Complex formation was blocked with anti-p50 antibody and was weakly inhibited with anti-p65 and anti-c-Rel (Fig. 6B, lanes 11–15 and 16–20). Thus the A1-κB site participates in PMA/PHA- and TNFα-induced expression of the MCP-1 gene, in agreement with data showing that this site of MCP-1 promoter is required for its TPA-induced expression (Ueda *et al.*, 1997).

HIV infection of U937 cells resulted in the appearance of a slower migrating protein–DNA complex, which was further identified by supershift analyses as the p65–p50 complex (Figs. 6A, lanes 2–5, and 6B, lanes 6–10). Interestingly, stimulation of HIV-infected U937 cells with PMA/PHA and TNFα revealed a delay in the appearance of the p50–p65 complex early after infection—days 4 and 6 (compare Fig. 6B, lanes 2 and 3, with lanes 7, 8, 12, and 13) and a complete inhibition of the PMA/PHA- or TNFα-induced NF-κB complexes identified in control cells (Fig. 6A, lanes 6–8 and 11–13). At later times—8 or 24 days—the HIV-induced NF-κB complex was detected, but not the PMA/PHA- or TNFα-induced complexes observed in uninfected cells (Fig. 6A, lanes 9–10 and 14–15). Both the delayed induction of the HIV-specific NF-κB complex and the inhibition of the PMA/PHA- and TNFα-induced NF-κB activity correlated with the inhibition of PMA/PHA- and TNFα-induced expression of MCP-1 mRNA observed after 4 and 6 days of HIV-1 infection (Figs. 2B and 3A). An identical pattern of inhibition of NF-κB activation and binding was observed when the A2-κB site of the MCP-1 distal promoter was used as a probe (data not shown), indicating the involvement of both sites in MCP-1 regulation.

Inhibition of NF-κB binding to the RANTES promoter

To determine if the RANTES promoter may be similarly regulated by HIV-induced inhibition of NF-κB binding at early times after HIV-1 infection, NF-κB activity was assessed using the −43 to −30 region of the RANTES promoter, which contains two adjacent NF-κB sites (Lin *et al.*, 1999). A similar pattern of PMA/PHA- and TNFα-induced NF-κB binding was observed (Fig. 7, lanes 1, 6, and 11) and similar inhibitory effects of HIV infection on NF-κB protein–DNA complexes were also detected (Fig. 7, compare lanes 2 to 5 with lanes 7 to 10 and 12–15). This observation suggests that identical mechanisms of inhibition of NF-κB binding activity may be responsible for the decrease in PMA/PHA- and TNFα-induced RANTES mRNA expression shown after 4 and 6 days of HIV infection (Fig. 2B).

DISCUSSION

The importance of chemokine gene expression during HIV infection has been emphasized by the finding that infection of CD4$^+$ T cells by M-tropic strains of HIV-1 is antagonized by the chemokines RANTES, MIP-1α, and MIP-1β, the natural ligands of CCR5 (Raport *et al.*, 1996). However, conflicting data regarding the influence of β-chemokines on HIV-1 replication in MDM and tissue

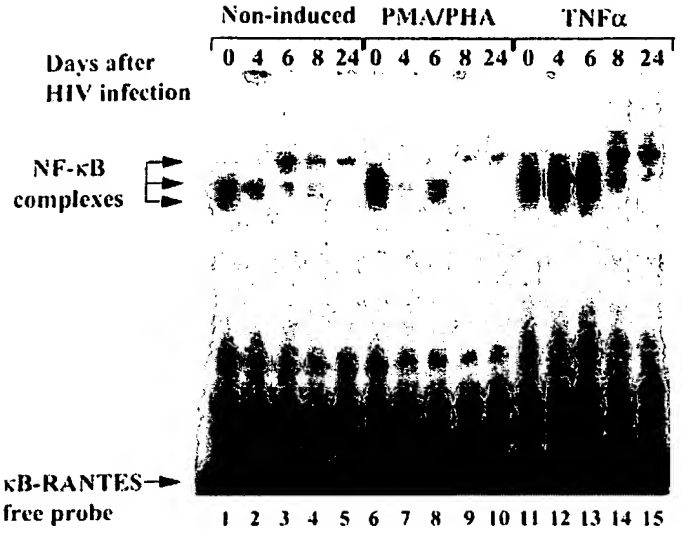

FIG. 7. Similar patterns of NF-κB binding activity observed with the NF-κB site of RANTES promoter. U937 myelomonoblastic cells were infected for 2 h with the HIV-1 strain IIIB. Before infection (lanes 1, 6, and 11) or at days 4 (lanes 2, 7, and 12), 6 (lanes 3, 8, and 13), 8 (lanes 4, 9, and 14), and 24 (lanes 5, 10, and 15) after infection, 5×10^6 cells were left unstimulated (lanes 1–5) or treated either with PMA (50 ng/ml) and PHA (10 ng/ml) (lanes 6–10) or with TNFα (10 ng/ml, lanes 11–15) for 8 h. Whole cell extracts (10 μg) were subjected to EMSA analysis using a [γ-^{32}P]ATP-labeled NF-κB RANTES probe. Arrows indicate the position of specific NF-κB complexes.

macrophages have been reported, with observations of enhancement, inhibition, or no effect (Schmidtmayerova *et al.*, 1996; Dragic *et al.*, 1996; Simmons *et al.*, 1997; Coffey *et al.*, 1997). The dichotomous effects of β-chemokines on HIV replication appear to depend on whether monocytes and MDM are exposed to chemokines before, simultaneously with, or after infection (Kelly *et al.*, 1998). Moreover, enhanced HIV-1 replication following inhibition of endogenous β-chemokines by neutralizing antibodies has been reported (Kinter *et al.*, 1996). The levels of chemokine expression and secretion therefore influence the ability of HIV-1 virus to replicate into target cells.

Using U937 monocytic cells and HIV-infected U937 cells as a model of chronic HIV infection, Kelly *et al.* reported an increased level of constitutive expression of the β-chemokines RANTES and MIP-1α (Kelly *et al.*, 1998). Chronic infection of myelomonocytic U937 or PLB985 cells produces constitutive NF-κB activity and recent data have shown that chronic and *de novo* HIV infection of monocytic cells results in selective activation of NF-κB (p50/p65) via the activation of the IKKβ subunit of the recently identified IκBα kinase (IKK) complex (DeLuca *et al.*, 1999; Asin *et al.*, 1999). Since several chemokine genes are regulated in part by NF-κB, it is likely that upregulation of constitutive and induced chemokine expression are due to an HIV-mediated increase in NF-κB transactivation in chronically infected cells (reviewed in Roulston *et al.*, 1995). Actually, NF-κB has been shown to constitute a potential activator of RANTES expression (Moriuchi *et al.*, 1997) and to be responsible for the serum- and endotoxin-mediated induction of MIP-1α expression in macrophages (Grove and Plumb, 1993). Similarly, TNFα- and PMA-induced expression of the MCP-1 gene is suggested to be mainly due to NF-κB activation (Freter *et al.*, 1996; Ping *et al.*, 1996; Ueda *et al.*, 1997).

In this paper, we examined chemokine gene expression during HIV infection of monocytic cell lines and primary monocytes/macrophages. PMA/PHA-induced transcription levels of MIP-1α and MIP1-β as well as IL-8 genes were increased in chronically HIV-infected U937 cells. The differential effect of HIV infection on chemokine expression was highlighted by the complete inhibition of PMA/PHA-induced transcription of MCP-1 and γ-IP10 in U9-IIIB cells, suggesting that HIV may bypass normal PMA/PHA- or TNFα-mediated induction of certain chemokine genes, such as MCP-1 and to a lesser extent γ-IP10. The inhibition of MCP-1 expression was confirmed at both mRNA and protein levels, during the course of *de novo* infection in U937 cells. Interestingly, inhibition was only transient, with the maximum decrease reached at 6–8 days after infection. In contrast to chronic infection, *de novo* infection of U937 cells produced a similar transient downregulation of MIP-1α and MIP-1β transcription, RANTES expression was decreased, and PMA/PHA-induced transcription of the IL-8 gene was unaffected. These discrepancies between chronic and *de novo* infection of U937 may reflect the *in vivo* complexity of HIV infection, the state of monocytic maturation, and the differentiation state at which the infection occurs. Interestingly, the similarities in MCP-1 and RANTES transcriptional inhibition induced by either HIV infection or *in vitro* differentiation of monocytes suggest a common mechanism that leads to MCP-1- and RANTES-specific repression, at least in monocytic cells. Finally, our data may provide an explanation of the functional alterations in chemotaxis associated with HIV infection of myeloid cells (reviewed in Roulston *et al.*, 1995).

By EMSA, we showed that HIV infection of U937 cells led, at early times after infection, to a delayed induction of the HIV-specific NF-κB binding complex, as well as to a complete inhibition of PMA/PHA- or TNFα-induced NF-κB binding activity to the NF-κB sites of the MCP-1 promoter. This decrease in DNA binding activity observed at 4 and 6 days after HIV-1 infection may thus explain the repression of PMA/PHA- and TNFα-induced MCP-1 transcription occurring at early times of infection. The EMSA data also suggest that identical mechanisms of NF-κB inhibition may be responsible for the decrease in PMA/PHA- and TNFα-induced RANTES expression at 4 and 6 days after HIV infection. Furthermore, our results associate the appearance of an HIV-specific NF-κB complex with the increase in MCP-1 and RANTES mRNA observed in unstimulated cells or at later times of infec-

tion (days 8 and 24). However, other mechanisms, such as posttranscriptional control, could be involved in virus-induced activation or repression of the MCP-1 and/or RANTES genes, since it has recently been shown that induced expression of RANTES by the respiratory syncytial virus (RSV) is mediated by increases in promoter activity as well as stabilization of RANTES mRNA (Koga *et al.*, 1999).

Transient inhibition of MCP-1 expression in U937 cells following HIV infection may constitute a novel mechanism by which HIV-1 escapes the influence of MCP-1 expression and subsequent CCR2 binding on virus entry/fusion or postentry events. Similarly, RANTES expression is also inhibited by HIV, at least in acute infection. Interestingly, MCP-1 and RANTES are targeted by another unrelated virus—human cytomegalovirus (HCMV)—which encodes a homologue of a CC chemokine receptor gene (US28) that is capable of binding MCP-1 and RANTES (Gao and Murphy, 1994). Depletion of these chemokines from the medium of HCMV-infected cells was shown to be partially due to continuous internalization of extracellular chemokine by expression of the US28 protein at the cell surface (Bodaghi *et al.*, 1998). The observation that MCP-1 accumulates in the cerebrospinal fluid of individuals with CMV encephalitis and recruits monocytic cells (Bernasconi *et al.*, 1996), thus affecting the ability of virus to persist and replicate in the brain, suggests that inhibiting MCP-1 and RANTES by US28 is crucial for CMV productive replication in the brain. Similarly, inhibition of chemokine secretion by HIV-1 may counteract the inhibitory effects of chemokines on viral infection. In contrast to HCMV, downregulation of MCP-1 by HIV-1 is due to transcriptional repression. Consequently, reexpression of MCP-1 during the course of HIV-1 infection of monocytic cells may alter virus spread in monocytes/macrophages.

MCP-1 chemokine receptor CCR2 is implicated in HIV infection by virtue of its ability to support infection by some strains *in vitro* (Doranz *et al.*, 1996; Rucker *et al.*, 1996; He *et al.*, 1997). Furthermore, a polymorphism in the CCR2 chemokine receptor in which Val 64 is replaced by Ile (CCR2-64I) has been associated with a 2- to 4-year delay in the progression to AIDS (Smith *et al.*, 1997). The relation between MCP-1 and HIV infection was further reinforced by the discovery that the CCR2b ligands MCP-1 and MCP-3 inhibited productive infection of PBMCs by both the CCR5 and CXCR4 strains of HIV-1 (Frade *et al.*, 1997; Schols *et al.*, 1997). However, the basis for the protective effects of CCR2-64I polymorphism and MCP-1/MCP-3 expression is currently unclear since only a few HIV-1 strains use CCR2b to infect $CD4^+$ cells (Doranz *et al.*, 1996). Recently, Lee *et al.* hypothesized that heterologous desensitization of CCR5 and CXCR4 signaling by the CCR2 receptor provides a link that might explain both the *in vivo* effects of the CCR2 gene variant and the antiviral activity of CCR2 ligands (Lee *et al.*, 1998). Heterologous desensitization of the RANTES response by MCP-1 was previously reported in human monocytic cells (Charo *et al.*, 1994). HIV-1 env can signal through CCR5 and CXCR4 (Davis *et al.*, 1997; Weissman *et al.*, 1997), and coreceptor signaling, although not required for env-mediated fusion or entry, can influence postentry events of virus replication in primary cells (Aramori *et al.*, 1997). Thus, MCP-1 expression may affect HIV infection via signaling through the CCR2 receptor and subsequent desensitization of the CCR5 and/or CXCR4 signaling pathway.

HIV-1 Tat protein induces CCR5 and CXCR4 expression in relation to Tat-enhanced infectivity of M- and T-tropic viruses, notably in monocytes/macrophages (Huang *et al.*, 1998). Furthermore, Tat, which is known to be chemoattractant for monocytes, has recently been demonstrated to interact with β-chemokine receptor CCR2 and CCR3, but not with CCR5 and to induce Ca^{2+} fluxes in monocytes (Albini *et al.*, 1998). Tat activation of CCR2 may stimulate the recruitment of chemokine expressing cells toward a productively infected cell, favoring the spread of HIV infection. These findings indicate that alterations in β-chemokine expression by HIV-1, and particularly of MCP-1 and RANTES, is essential for the establishment of HIV-1 infection.

MATERIALS AND METHODS

Cell culture and isolation of primary monocytes and lymphocytes

Myelomonoblastic U937 as well as U9-IIIB cells, infected with HIV-1 strain IIIB, were maintained in RPMI 1640 (GIBCO, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 10 μg/ml of gentamicin (Shering Canada, Pointe-Claire, Canada). Cells were stimulated with either 50 ng/ml of PMA (Sigma Chemical Co., St Louis, MO) and 100 ng/ml of PHA (Sigma) or 10 μg/ml of TNFα (R&D Systems, Minneapolis, MN). Peripheral blood mononuclear cells were obtained from HIV-negative, healthy donors, separated by Ficoll/Hypaque (Pharmacia, Upsalla, Sweden) centrifugation as described previously (Conti *et al.*, 1997), and seeded on FBS-coated plastic tissue culture plates (Mosier, 1984). After an overnight incubation at 37°C in RPMI 1640 supplemented with 30% FBS, nonadherent cells, which mainly constitute lymphoid cells, were removed and cultured in 10% FBS RPMI 1640. Adherent cells were extensively washed and cultured 10% FBS RPMI 1640. At 1, 3, 6, and 9 days, adherent cells were recovered and analyzed. We refer to monocytes as cells processed 24 h after seeding, while 3-, 6-, and 9-day cultured adherent cells were considered macrophages.

HIV infection of U937 and reverse transcriptase assay

U937 cells were incubated with concentrated HIV-1 strain IIIB, a clone derived from the molecular HXB2D (Fisher *et al.*, 1985) at a m.o.i. of 0.3 (viral concentration of 10^6 PFU/ml) in a serum-free medium, for 2 h at 37°C. After virus exposure, the medium was replaced. Samples were obtained every 2 or 3 days for assay of reverse transcriptase (RT) activity. RT activity was determined by incubating 50 μl of cell supernatants (precleared by centrifugation at 3000 rpm for 30 min at 4°C) with a reaction mixture containing [^{3}H]dTTP (2 Ci/mmol, Amersham, Cleveland, OH) in Tris–HCl (pH 7.9) for 20 h at 37°C as described (Lee *et al.*, 1987). Radiolabeled nucleotides were precipitated on GF/A Whatmann filters by using cold 10% trichloroacetic acid and 95% ethanol. Incorporated activity was measured by liquid scintillation.

RNA extraction and RNase protection assay

Total RNA from U937 and U9-IIIB cells was extracted using RNeasy Mini kit (QIAGEN, Valencia, CA). RNA from primary monocytes/macrophages was prepared as described (Ausubel *et al.*, 1989). Briefly, cells were lysed in a guanidium thiocyanate homogenization buffer (4.0 M guanidium thiocyanate, 0.1 M Tris–HCl, pH 7.5, 1% β-mercaptoethanol) and the cell lysate was homogenized by passage through a syringe. Sodium lauryl sarcosinate (0.5%) was added and the suspension was centrifuged at 5000 rpm for 10 min at room temperature. The lysate was layered on a cushion of CsCl (5.7 M). RNA of higher density (>1.8 g/ml) than that of other cellular components was separated by centrifugation for 24 h at 35,000 rpm. The RNA pellet was washed, resuspended in TE/SDS, reprecipitated in ethanol, centrifuged, washed, dried, and resuspended in sterilized H_2O. Total RNA (5 μg) was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit as recommended by the manufacturer (PharMingen, San Diego, CA). Labeled fragments protected from RNase digestion and corresponding to MCP-1 mRNA were quantified using the NIH Image 1.60 software package. Values were normalized to the L32 (housekeeping gene) mRNA levels and plotted as MCP-1/L32 mRNA ratios. Similar results were obtained in three independent experiments when the MCP-1 mRNA signal was normalized according to the GAPDH mRNA.

ELISA detection assay

Concentration of secreted MCP-1 protein was determined from 50 μl of the supernatants of cell cultures (precleared by centrifugation at 3000 rpm for 30 min at 4°C) using the Human Monocyte Chemoattractant Protein-1 (hMCP-1) ELISA kit (BioSource Int., Camarillo, U.S.A.) as described by the manufacturer.

EMSA

Whole cell extracts were prepared as previously described (Kwon *et al.*, 1998) and 10 μg was subjected to EMSA by using ^{32}P-labeled probes, DNA binding buffer (10 mM HEPES, pH 7.9, 2% glycerol (v/v), 40 mM KCl, 1 mM EDTA, pH 8.0, 0.2 mM $MgCl_2$, 1 mM DTT, 0.05 mM PMSF), 0.2% NP-40, 0.5 μg of BSA, and 1 μg of poly(dI:dC). Incubation was performed for 30 min at room temperature. Oligonucleotides used were (−2645 to −2626) A1-κB of MCP-1 promoter, 5′-GATCTGGGAACTTCCAAAGC-3′; (−2616 to −2597) A2-κB of MCP-1 promoter, 5′-AGAGTGGGAATTTCCACTCA-3′; and (−43 to −30) NF-κB of RANTES promoter, 5′-ACTCCCCTTAGGGGATGCCCCTCAA-3′. The resulting protein–DNA complexes were resolved on 5% polyacrylamide (37.5:1) TBE 0.25× gels and exposed for 16 h. To demonstrate the specificity of protein–DNA complex formation, a 100-fold molar excess of unlabeled oligonucleotide was added to the extracts before adding labeled probe. Supershift analysis was performed by preincubating 1 μl of anti-p65, anti-p50, and anti-c-Rel antibodies (Santa Cruz Biotechnology Inc.) with extracts and binding buffer for 30 min at 4°C before adding probe.

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Sensitivity of human immunodeficiency virus infection to various α, β and γ chemokines

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Examination of a large panel of chemokines indicates that in addition to RANTES, MIP-1α and MIP-1β, the β-chemokine MCP-2 and, to a lesser extent, the γ-chemokine lymphotactin also show anti-human immunodeficiency virus (HIV) activity in cell culture. The amount of chemokine needed to suppress HIV replication by ≥50% was generally greater (≥250 ng/ml) than that required for inhibition of virus infection by RANTES, MIP-1α and MIP-1β. The β-chemokine MCP-3 was found to enhance the replication of both non-syncytium-inducing (NSI) and syncytium-inducing (SI) viruses at high concentrations (0·5–5 μg/ml). In contrast to a previous report, macrophage-derived chemokine was not found to inhibit HIV replication of either NSI or SI viruses, but at low concentrations enhanced NSI virus replication. When small amounts of RANTES or MCP-2 were added together with high concentrations of non-inhibitory chemokines, the anti-HIV effects were countered. Information on chemokines that affect HIV infection could be useful for future therapeutic strategies.

The β-chemokines RANTES, MIP-1α and MIP-1β have been shown to inhibit the infection of cultured peripheral blood mononuclear cells (PBMC) by non-syncytium-inducing (NSI) isolates of HIV (Cocchi *et al.*, 1995). This finding, along with the identification of the receptor for stromal-derived factor-1 as an HIV-1 coreceptor (Feng *et al.*, 1996), led to observations that several chemokine receptors can act as coreceptors for HIV infection (reviewed by Berger, 1997). Recently, macrophage-derived chemokine (MDC) (Pal *et al.*, 1997) and monocyte chemotactic protein-2 (MCP-2) (Gong *et al.*, 1998) have been identified as other β-chemokines that can block HIV infection. These chemokines can exert their anti-HIV activity either by interfering with the binding of the virus to its coreceptor or by down-regulating the receptor expression on the cell surface (Trkola *et al.*, 1998).

Our present studies with a large panel of chemokines indicate that most had no effect on HIV infection. RANTES, MIP-1α and MIP-1β, as well as MCP-2, and to a lesser extent, lymphotactin, significantly inhibited replication of certain primary isolates of HIV-1. MCP-3 and MDC enhanced virus replication. In addition, we found that the chemokine-mediated antiviral effect of RANTES and MCP-2 could be prevented, in part, by the addition of another chemokine with different biological activity.

For these studies $CD4^+$ cells from seronegative donors were isolated from Ficoll–Hypaque-separated PBMC using anti-CD4 antibody-coupled immunomagnetic beads (Dynal) (Mackewicz *et al.*, 1991). More than 95% were $CD4^+/CD3^+$ cells (less than 1% $CD8^+$ cells) as assessed by flow cytometry (Landay *et al.*, 1993). Cell cultures were grown in RPMI 1640 medium (BioWhittaker) supplemented with 10% heat-inactivated (56 °C, 30 min) foetal calf serum (Gemini Bioproducts), 100 U/ml recombinant IL-2 (Glaxo-Wellcome), 2 mM L-glutamine and 1% antibiotics.

The four primary HIV-1 isolates used (designated SV, NB, EM and ALA-22) were obtained from clinically healthy HIV-infected subjects. The biological phenotype of SV, NB and ALA-22 was non-syncytium-inducing as determined in MT-2 cells. These viruses have been cultivated in the laboratory for less than 2 months and only in PBMC. Their infection is blocked by RANTES, MIP-1α and MIP-1β. The SF2 strain of HIV-1, isolated from a patient with candidiasis (Levy *et al.*, 1984), and the primary isolate, EM, have a tropism for T-cell lines *in vitro*. They are syncytium-inducing (SI), non-macrophage-tropic viruses resistant to the inhibitory effects of the β-chemokines MIP-1α, MIP-β and RANTES (Mackewicz *et al.*, 1996). The $TCID_{50}$ of each virus was determined in PBMC as described (McDougal *et al.*, 1985).

The purified $CD4^+$ T-cells were stimulated with phytohaemagglutinin (PHA, 3 μg/ml; Sigma) for 3 days, washed and infected with 10–40 $TCID_{50}$ of HIV-1 per 10^6 cells as described (Barker *et al.*, 1998). After incubation for 2 h, cells were washed, and plated at 5×10^5 cells per well in duplicate or triplicate in 48-well plates (Becton Dickinson). Varying concentrations of recombinant chemokines were added and the cultures were

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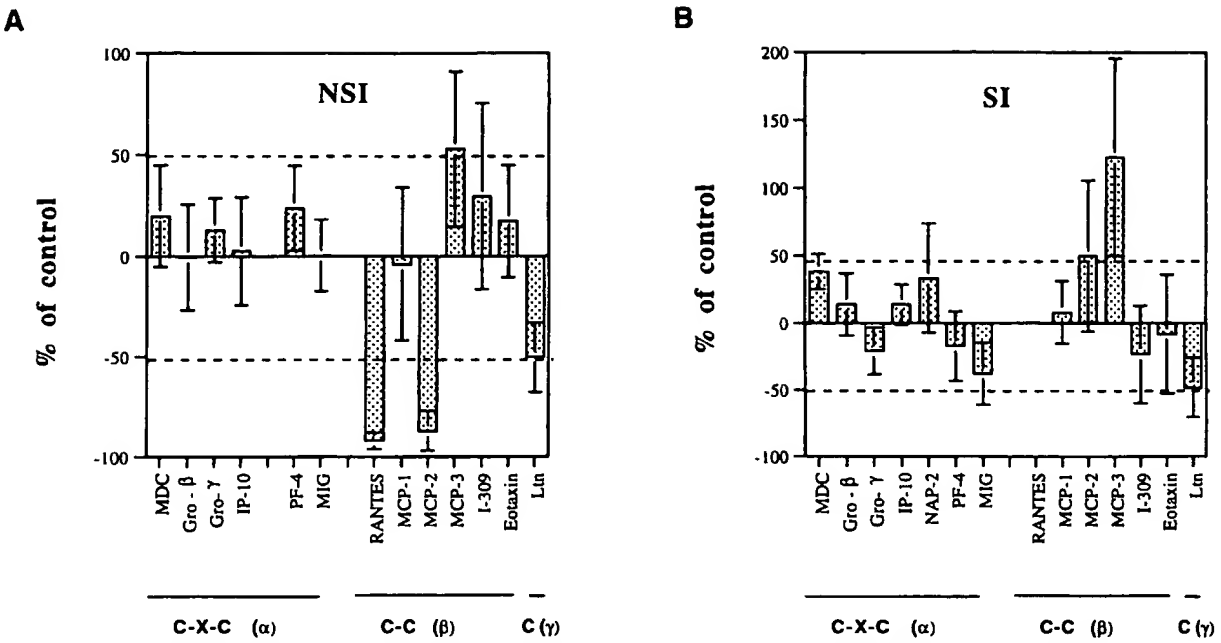

Fig. 1. Effect of chemokines on HIV-1 replication in $CD4^+$ lymphocytes. Non-syncytium-inducing (NSI) viruses (SV, ALA-22, NB) (A) or syncytium-inducing (SI) virus isolates (EM, SF2) (B) were inoculated onto PHA-stimulated $CD4^+$ T-cells at an input of 10–40 $TCID_{50}$ per 10^6 cells. Two hours after infection, cells were washed and plated at about 10^6 cells per well in duplicate wells of a 48-well plate in the presence of different concentrations of chemokines. Results obtained using 0·5–1 μg/ml of chemokines are shown (with the exception of MCP-1 with NSI and SI strains, and MCP-3 with SI strains: 5 μg/ml was used). Cultures were maintained for 6–10 days and were replenished with fresh chemokine-containing medium every 2–3 days. Virus replication was measured in the collected supernatants by RT assays (Hoffman *et al.*, 1985). The effect of each chemokine on virus replication is shown as a percentage relative to that obtained with infected $CD4^+$ T-cells inoculated with virus alone. The results are representative of at least two separate experiments per chemokine, with at least two different NSI or SI isolates. The results were confirmed with $CD4^+$ cells from different donors. Suppression or enhancement was considered present if virus replication was reduced or increased by ⩾ 50%, respectively. Suppression by RANTES was observed with some NSI viruses at 5–50 ng/ml; MIP-1α, MIP-1β and MCP-2 generally inhibited NSI virus replication at ⩾ 100 ng/ml (data not shown). Lymphotactin suppressed NSI viruses sometimes at 1 μg/ml and consistently suppressed both NSI and SI viruses at 2 μg/ml. Enhancement of NSI and SI viruses by MCP-3 was sometimes observed at 200–2000 ng/ml; it was consistently noted at 5 μg/ml. With MDC, enhancement was observed with 10–100 ng/ml. In some experiments, depending on the donor $CD4^+$ cells used, enhanced HIV replication of NSI strains of HIV was observed at ⩾ 1 μg/ml eotaxin and ⩾ 3 μg/ml MCP-1. Sometimes inhibition of HIV replication was observed with Gro-β at ⩾ 100 ng/ml. NAP-2 did not show any effect with NSI strains in one experiment. IL-8, interleukin 8; Gro, growth-related oncogene; IP10, interferon-inducible protein 10; NAP-2, neutrophil-attracting peptide 2; PF-4, platelet factor 4; MIG, monokine induced by γ-interferon; RANTES, regulated on activation normal T-cell expressed and secreted; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine.

passaged every 2–3 days for up to 1 week. Cultures were replenished with fresh medium and chemokine at each passage. The culture fluids were monitored for virus replication by measurement of reverse transcriptase (RT) activity (Hoffman *et al.*, 1985).

The recombinant chemokines studied were used at concentrations ranging from 50 ng to 5000 ng/ml and were provided by Michael Luther, Glaxo-Wellcome. All were obtained from R&D Systems, except for NAP-2, MIG, PF4 and lymphotactin, which were obtained from Peprotech. The recombinant MDC proteins evaluated came from both R&D Systems and Peprotech. The range of concentrations of chemokines tested for anti-HIV activity corresponded approximately to 0·5–100 ED_{50} of chemotactic activity (as noted by the company providing the reagents). Results were analysed for statistical significance by the Mann–Whitney U-test.

Fig. 1 shows the effect of 0·5–5 μg/ml of a large panel of commercially available recombinant chemokines. For most of the cytokines, the effect did not vary over a large range of concentrations (50 ng–5 μg). They caused a < 20% difference in HIV replication when compared with the control-infected culture, regardless of the virus used (Fig. 1). Besides RANTES, MIP-1α and MIP-1β, only MCP-2 and lymphotactin produced an inhibitory effect within the range of concentrations tested (Fig. 2). With MCP-2 and NSI viruses, up to a 90% decrease in virus replication was observed with ⩾ 1 μg/ml, and 70% with ⩾ 0·25 μg/ml (Figs 1 and 2). No substantial inhibitory effect of this chemokine on SI viruses (Fig. 2) was noted. Lymphotactin at a concentration of 1 μg/ml or more reduced the replication of both NSI and SI viruses by at least 50% (Fig. 2). In some experiments, Gro-β inhibited infection by SI viruses, but this finding was not consistently seen.

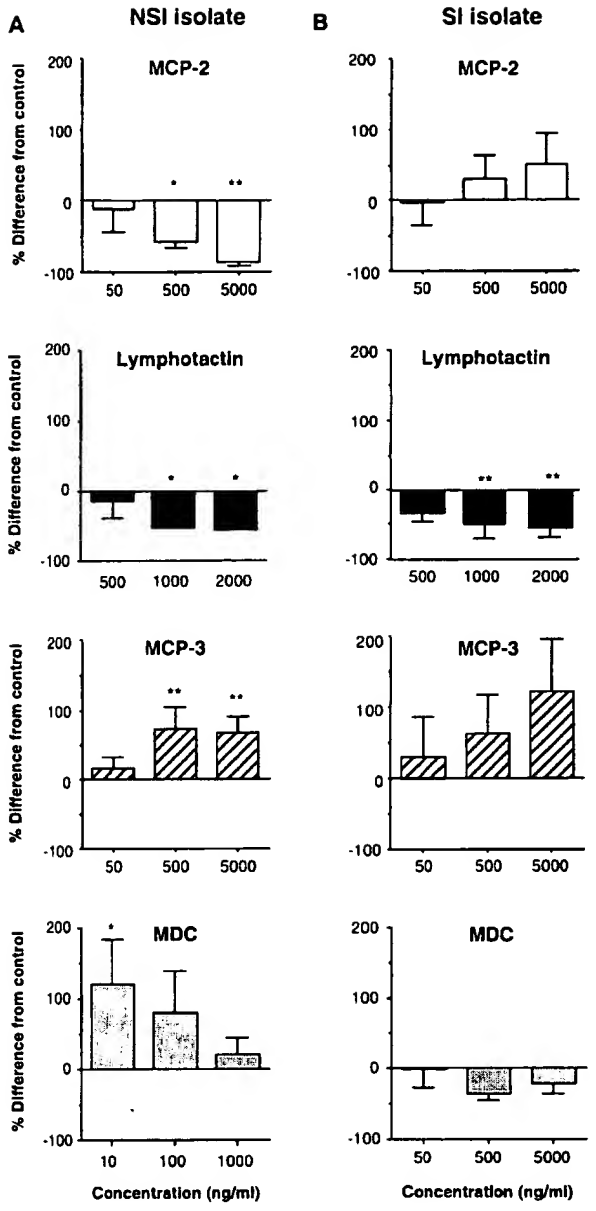

Fig. 2. Effect of MCP-2, lymphotactin, MCP-3 and MDC on HIV replication. PHA-stimulated CD4+ lymphocytes were inoculated with either the SV NSI isolate (A) or the SF2 SI isolate (B) at 10–20 $TCID_{50}$ per 10^6 cells. After 2 h, cells were washed and plated at 5×10^5 cells per well in triplicate in a 48-well plate in the presence of different concentrations (ng/ml) of recombinant MCP-2, lymphotactin, MCP-3 and MDC. Cultures were maintained for 7 days and replenished with fresh chemokine-containing medium every 2–3 days. Virus replication was measured in the collected culture supernatants by RT assays (Hoffman *et al.*, 1985). The number of replicates in the control and chemokine-treated group varied between 3–7 and 2–4 samples, respectively. The results reflect the extent of virus replication at day 7 expressed as a percentage relative to that obtained from control-infected CD4+ cells not receiving the chemokine. Virus replication in control cultures ranged between 100×10^5 and 300×10^5 c.p.m./ml of RT activity. The results are representative of five separate experiments, three different NSI viruses (SV, NB and ALA-22) and two SI isolates (EM, SF2). **, $P \leqslant 0{\cdot}01$; *$P \leqslant 0{\cdot}05$.

In contrast to the above observations, the β-chemokine MCP-3 consistently enhanced replication of both NSI and SI viruses when used at high concentrations (5 μg/ml) and at a lower concentration (0·5 μg/ml) with the NSI viruses evaluated (Figs 1 and 2). This effect was statistically significant for NSI viruses ($P < 0{\cdot}01$) and close to significant for SI viruses (5 μg/ml; $P < 0{\cdot}08$). MCP-1, a β-chemokine related to MCP (Adams & Lloyd, 1997), showed no substantial effect on replication of either NSI or SI isolates (Fig. 1). An enhancement of HIV infection was sometimes noted with MCP-1 and eotaxin with NSI isolates (Fig. 1). Surprisingly, MDC, at low concentrations, was also found to have an enhancing effect on the replication of NSI (P, 0·05), but no significant effect on SI isolates. As much as 200% increases in HIV replication were observed at 10 ng/ml MDC (Fig. 2). The variations in effects of chemokines on HIV replication (Figs 1 and 2) probably reflect differences in the $CD4^+$ cells used. In contrast to one report (Kinter *et al.*, 1998) we did not see enhancement of SI isolates by RANTES, MIP-1α and MIP-1β, but we did not pretreat cells with the chemokines nor use suboptimal concentrations of virus.

Several chemokines share receptors (Adams & Lloyd, 1997) and therefore may compete for the same receptor. By other mechanisms, the effect of one chemokine may dominate over another. In evaluating this possibility, RANTES at a concentration (5 ng/ml) that suppressed replication of NSI viruses (SV or NB) by 70% was mixed with MCP-3 (1000 ng/ml); the inhibition of virus production was abrogated. In another study, MCP-3 (2000 ng/ml) prevented inhibition of virus replication by a low concentration of RANTES (20 ng/ml). At higher concentrations of RANTES (80 ng/ml) this effect of MCP-3 was not observed. In addition, whereas MCP-2 (500–1000 ng/ml) inhibited replication of the NSI virus, ALA-22, the addition of MCP-1 (1000 ng/ml) prevented this activity.

These studies indicate that, in addition to the chemokines already known to have anti-HIV properties, MCP-2 and to a lesser extent, lymphotactin, showed consistent inhibition of HIV replication (Figs 1 and 2). As with MIP-1α, MIP-1β and RANTES, the effect of MCP-2 (at $\geqslant$ 1 μg/ml) was limited to NSI isolates. These results support findings recently reported (Gong *et al.*, 1998). Lymphotactin was effective in reducing virus replication of both NSI and SI isolates. However, high doses ($\geqslant$ 1 μg) of lymphotactin were necessary to show a substantial (55%) decrease in virus production (Fig. 2).

CCR5 and CXCR4 are the primary coreceptors for macrophage-tropic and T-cell-tropic HIV-1 strains, respectively (for a review see Berger, 1997). In addition, certain virus strains can use the CC-chemokine receptors CCR-1, CCR2b and CCR3 (Berger, 1997; Frade *et al.*, 1997). All the MCP recognize CCR2b (the chemokine receptor to which CCR5 is most closely related: 76% identity) (Rucker *et al.*, 1996), whereas CCR3 serves as a receptor for RANTES, MIP-1α, MIP-1β, eotaxin, MCP-2, MCP-3 and MCP-4 (Adams & Lloyd, 1997). Nevertheless, despite the overlap of receptor

usage among the MCPs, only MCP-2, probably because it can bind to CCR5 (Gong *et al.*, 1998), showed a reproducible inhibitory effect on replication of the NSI isolates evaluated (Figs 1 and 2).

Our results also indicated that, at certain concentrations, MCP-3 and MDC can enhance virus replication in $CD4^+$ cells (Figs 1 and 2). These findings contrast with a previous report showing inhibitory activity of MCP-3 on replication of SI isolates of HIV-1 in PBMC (Schols *et al.*, 1997), and MDC on both NSI and SI HIV isolates (Pal *et al.*, 1997). We did not detect any inhibition of HIV production by either of these chemokines. Recently, MDC was shown to be a ligand for CCR-4 but not CCR-1, CCR-3 or CCR-5 (Yoshida *et al.*, 1998). Thus, MDC would not be expected to compete for HIV binding to a virus coreceptor. However, these different observations may be due to the experimental conditions used (e.g. PBMC instead of $CD4^+$ cells), the various concentrations of chemokine evaluated or the source of the chemokine. Nevertheless, the recombinant MDC we used came from two sources (R&D Systems and Peprotech) and had chemotactic activity. The reported antiviral activity of MDC has also not been confirmed by other studies in which recombinant MDC was used (Lee *et al.*, 1998). The maturation of MDC, resulting in NH_2-terminal modification, could influence its receptor binding and activation profile. This possibility could account for the conflicting results obtained with the recombinant MDC versus the purified natural product originally used (Pal *et al.*, 1997).

The mechanisms underlying chemokine-mediated anti-HIV activity may involve competition for receptor occupancy and/or down-regulation of the receptor (Trkola *et al.*, 1998). Conceivably, MCP-2 could exert its inhibitory activity on NSI isolates by competitive binding to CCR5, which does not occur with the other MCPs; lymphotactin-mediated inhibition of NSI and SI strains could involve mechanisms different from competition for the receptors. In terms of enhancement, the increase in HIV replication observed with the addition of some chemokines (Figs 1 and 2) may result from upregulation of certain virus receptors as reported recently (Dolei *et al.*, 1998). These possibilities are under study.

Chemokines may have opposite regulatory effects and therefore specific combinations of chemokines could result in qualitatively different effects on HIV replication than that observed when single chemokines are used alone. In this regard, we have found that chemokines, devoid of any inhibitory activity on HIV, may affect inhibition induced by other chemokines. In particular, the concentration of RANTES or MCP-2 required to produce a certain level of inhibition must be increased when these chemokines are present in combination with MCP-3 and MCP-1, respectively.

This study presents further information on the potential role of chemokines in blocking HIV infection. Most chemokines do not appear to substantially affect the levels of HIV replication in $CD4^+$ T-cells. Moreover, levels of those chemokines that inhibit HIV infection were higher than expected under normal physiological conditions. Nevertheless, we cannot exclude the possibility that these chemokines play some role in HIV pathogenesis, perhaps via inflammatory responses or by interfering with HIV adaptation to certain coreceptors. Further understanding of these cytokines might assist in the development of novel antiviral strategies that are capable of blocking infection by both macrophage-tropic and T-cell line-tropic isolates.

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Monocyte Chemotactic Protein-2 Activates CCR5 and Blocks CD4/CCR5-mediated HIV-1 Entry/Replication*

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Human immunodeficiency virus, type I (HIV-1) cell-type tropism is dictated by chemokine receptor usage: T-cell line tropic viruses use CXCR4, whereas monocyte tropic viruses primarily use CCR5 as fusion coreceptors. CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated on activation normal T cell expressed and secreted) inhibit CD4/CCR5-mediated HIV-1 cell fusion. MCP-2 is also a member of the CC chemokine subfamily and has the capacity to interact with at least two receptors including CCR-1 and CCR2B. In an effort to further characterize the binding properties of MCP-2 on leukocytes, we observed that MCP-2, but not MCP-1, effectively competed with MIP-1β for binding to monocytes, suggesting that MCP-2 may interact with CCR5. As predicted, MCP-2 competitively inhibited MIP-1β binding to HEK293 cells stably transfected with CCR5 (CCR5/293 cells). MCP-2 also bound to and induced chemotaxis of CCR5/293 cells with a potency comparable with that of MIP-1β. Confocal microscopy indicates that MCP-2 caused remarkable and dose-dependent internalization of CCR5 in CCR5/293 cells. Furthermore, MCP-2 inhibited the entry/replication of HIV-1ADA in CCR5/293 cells coexpressing CD4. These results indicated that MCP-2 uses CCR5 as one of its functional receptors and is an additional potent natural inhibitor of HIV-1.

Members of the seven-transmembrane chemokine receptor superfamily have been identified as co-receptors for HIV-1[1] infection (1–8). HIV-1 cell type tropism seems to be dependent on chemokine receptor usage, and T-cell line tropic viruses use CXCR4, whereas monocyte tropic viruses primarily use CCR5 as fusion coreceptors. A minority of HIV-1 strains may also use other CC chemokine receptors as their fusion co-factors. Dual tropic HIV-1 strains presumably interact with more than one type of chemokine receptor (5, 8). The CC chemokines MIP-1α, MIP-1β, and RANTES were able to inhibit the entry of monocyte tropic viruses (9), whereas the CXC chemokine SDF-1 abrogates CXCR4-mediated fusion by T lymphotropic HIV-1 strains (6, 7).

Monocyte chemotactic protein (MCP)-2 is a CC chemokine co-purified with MCP-1 and MCP-3 from human osteosarcoma cells (10–12). It shares over 60% amino acid identity with MCP-1 and MCP-3 and has about 30% identity with the CC chemokines MIP-1α, RANTES, and MIP-1β (10–12). MCP-2, similar to MCP-3, is chemotactic for and activates a wide variety of inflammatory cells, including monocytes, T lymphocytes, NK cells, basophils, mast cells, and eosinophils (12), but differs from MCP-1, which is not active on eosinophils (13). We recently reported that MCP-2 uses CCR1 and CCR2B as its functional receptors (14), which may account for its action on a greater variety of target cells. In the course of studies on leukocyte activation by MCP-2, we observed that MCP-2 could competitively inhibit the binding to monocytes of ^{125}I-MIP-1β, a CC chemokine that is believed thus far to exclusively use CCR5 as a functional receptor on human leukocytes (15–17). This prompted us to further investigate the effect of MCP-2 on cloned CCR5. We report that MCP-2 is also an efficient ligand for CCR5 and a potent inhibitor of CD4/CCR5-mediated HIV-1 entry/replication.

EXPERIMENTAL PROCEDURES

Chemokines—Recombinant human (rh) MCP-2 and other chemokines were purchased from PeproTech Inc. (Rocky Hill, NJ). Radioiodinated MCP-2 was a kind gift from Dr. G. Brown (NEN Life Science Products). Other radioiodinated chemokines were purchased from NEN. All radioiodinated chemokines have a specific activity of 2200 Ci/mmol.

Cells—Human peripheral blood monocytes were isolated from normal donors (National Institutes of Health Clinical Center, Transfusion Department, Bethesda, MD) with an iso-osmotic Percoll (Pharmacia Biotech Inc.) gradient as described elsewhere (18). The monocyte preparations were >90% pure. The 293 human embryonic kidney epithelial cell line stably transfected with FLAG-tagged CCR5 (CCR5/293) was generated and grown in monolayers as described (17).

Binding Assays with Radiolabeled Chemokines—Binding assays were performed by using a single concentration of radiolabeled chemokines in the presence of increasing concentrations of unlabeled ligands as described previously (14, 18). Cells (2×10^6/sample for monocytes and 1×10^6/sample for CCR5/293 cells) were suspended in 200 μl of modified binding medium composed of RPMI 1640, 1 mg/ml bovine serum albumin, 25 mM HEPES, and 0.05% sodium azide and incubated in duplicates at room temperature for 40 min. After incubation, the cells were pelleted through a 10% sucrose/phosphate-buffered saline cushion, and the radioactivity associated with cell pellets was determined in a γ-counter (Clinigamma-Pharmacia, Gaithersburg, MD). Experiments were also performed at 4 °C in the absence of sodium azide and yielded similar binding and competition curves as obtained at room temperature. The binding data were then analyzed with a Macintosh computer program LIGAND (P. Munson, Division of Computer Research and Technology, NIH, Bethesda, MD). The degree of competition for binding by unlabeled chemokines was calculated as follows: % competition for

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type I; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; rh, recombinant human; PMA, phorbol 12-myristate 13-acetate; IL, interleukin; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction.

binding = 1 − (cpm obtained in the presence of unlabeled ligand/cpm obtained in the absence of unlabeled ligand) × 100.

Chemotaxis Assay—The migration of HEK 293 cells transfected with cDNA clones was assessed by a 48-well microchamber technique (14, 19). Different concentrations of chemokines were placed in the lower wells of the chamber. The CCR5/293 cells (50 μl, 0.5–1 × 10^6/ml) were loaded in the upper wells. The lower and upper wells were separated by a polycarbonate filter (polyvinylpyrrolidone-free, 10-μm pore size; Poretics, CA) precoated with 20 μg/ml mouse collagen type IV or 50 μg/ml collagen type 1 for 2 h at 37 °C. The chamber was incubated at 37 °C for 5 h in humidified air with 5% CO_2. At the end of the incubation, after removal of nonmigrating cells, the filter was fixed and stained with Diff-Quik (Biochemical Sciences, NJ). The cells migrating across the filter were counted in three high power fields under light microscopy in triplicates with all samples coded. The chemotaxis index was calculated as the number of cells migrating to chemokines/number of cells migrating to medium. The significance of the difference between test and control groups was analyzed by paired Student's *t* test.

Confocal Microscopy—CCR5/293 cells were pretreated for 3 h at 37 °C with PMA (100 nM), RANTES, IL-8 (120 nM), or different concentrations of MCP-2. The cells were centrifuged on to slides and permeabilized using 0.15% saponin in phosphate-buffered saline. The slides were then stained with an anti-FLAG monoclonal antibody (M1, Kodak, New Heaven, CT) followed by incubation with FITC-labeled goat anti-mouse IgG F(ab')$_2$ fragments. Slides were examined using a Zeiss 310 Confocal Laser Scanning Microscope (Carl Zeiss). Nomarski and FITC (488 nm, green) images were prepared for each specimen, and fluorescent images were superimposed on Nomarski images.

HIV-1 Inhibition Assay—CCR5/293 cells were co-transfected using electroporation with a CD4 expression vector (fragment excised from pTB4, NIH AIDS Research and Reference Reagent Program, Bethesda, MD) and cloned into a plasmid vector, pSVZeo (Invitrogen, CA). 24 h after electroporation, the cells (CCR5/CD4/293) were plated at a density of 10^5/well in 24-well plate for 24 h. The culture media were removed, and the cells were preincubated for 30 min with 800 ng/ml recombinant human MCP-2 or RANTES. The monocyte tropic HIV-1$_{ADA}$ was added at a final multiplicity of infection between 1 and 0.1. Cultures were continued for 24 h and washed, and total genomic DNA was isolated and purified (20). HIV-1 entry/reverse transcription was monitored by polymerase chain reaction (PCR) using primers specific for HIV-1gag region to detect late reverse transcription products (21). Two primers are as follows: M667 corresponds to nucleotide positions 496–516 in the HIV-1_{JR-CSF} sequence; M661 (antisense) corresponds to the positions 695–672. PCR products were resolved on 2% agarose gels and photodocumented after ethidium bromide staining. No PCR products were detected in CCR5/CD4/293 cells infected by the lymphocyte tropic virus HIV-1RF nor in CCR5/293 cells without CD4 coexpression incubated with HIV-1$_{ADA}$.

RESULTS AND DISCUSSION

Recombinant human MCP-2 is a potent monocyte chemoattractant and has been shown to activate at least two promiscuous CC chemokines receptors, CCR1 and CCR2B (14). The binding of ^{125}I-labeled MCP-2 to human monocytes was of high affinity and was efficiently competed by MCP-1, which is a ligand for CCR2B. MCP-3, which uses both CCR1 and CCR2B, also efficiently competed with MCP-2 for binding to monocytes. MIP-1α and RANTES, two ligands for both CCR1 and CCR5, partially competed with MCP-2 for binding to monocytes, whereas MIP-1β, which only uses CCR5, had no effect (14). We further observed that although unlabeled MIP-1β did not competitively inhibit MCP-2 binding to monocytes, the binding of ^{125}I-MIP-1β was completely inhibitable by unlabeled MCP-2 (IC_{50} = 2.9 nM *versus* 1.7 nM for unlabeled MIP-1β itself, Fig. 1). In contrast, the binding of ^{125}I-MIP-1β to monocytes was only partially but significantly displaced by unlabeled MCP-1 at high concentrations (IC_{50} = 425 nM), in agreement with our earlier observations (22). There was no significant difference between the competition curves yielded at room temperature (Fig. 1*A*) and at 4 °C (Fig. 1*B*). The uni-directional inhibition of MIP-1β binding to monocytes by MCP-2 prompted us to investigate whether MCP-2, in addition to using CCR1 and CCR2B as functional receptors, also uses CCR5. HEK293 cells stably expressing CCR5 (CCR5/293) showed a high level of specific

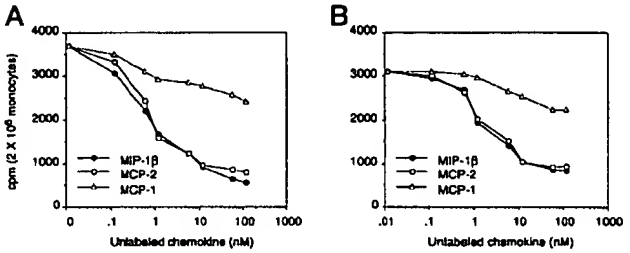

FIG. 1. **Competition of ^{125}I-MIP-1β binding to human monocytes by MCP-2.** Aliquots of monocytes (2 × 10^6/200 μl) were incubated with 0.12 nM of ^{125}I-MIP-1β in the presence of different concentrations of unlabeled chemokines. The binding assays were performed at room temperature (*A*) or at 4 °C in the absence of 0.05% sodium azide (*B*). Curves shown are from one experiment. Six experiments were performed yielding similar results. Unlabeled MCP-1 at 10 nM and more showed partial but significant competition of ^{125}I-MIP-1β binding ($p < 0.05$ compared with binding in the absence of unlabeled ligand, Student's *t* test).

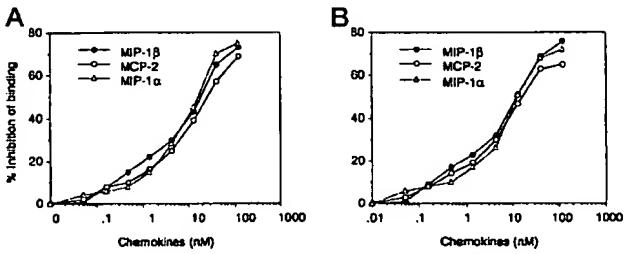

FIG. 2. **Competition of ^{125}I-MIP-1β binding to CCR5/293 cells by MCP-2.** Aliquots of CCR5/293 cells (1 × 10^6/200 μl) were incubated with 0.12 nM of ^{125}I-MIP-1β in the presence of increasing concentrations of unlabeled chemokines. The binding assays were performed at room temperature (*A*) or at 4 °C in the absence of sodium azide (*B*). Results from one experiment out of five performed are shown.

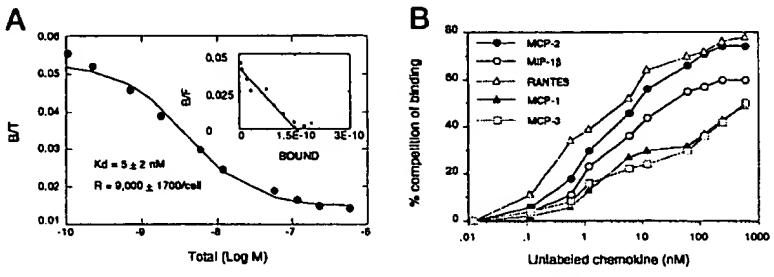

FIG. 3. **Binding of ^{125}I-MCP-2 to CCR5/293 cells and competition by CC chemokines.** *A*, Scatchard analysis. *B*, Cross-competition. CCR5/293 cells were incubated with 0.12 nM of ^{125}I-MCP-2 in the presence of different concentrations of unlabeled MCP-2. The binding assays were performed at room temperature. The data are from one representative experiments out of three performed and were analyzed using the Macintosh computer program LIGAND. MCP-1 and MCP-3 at 10 nM and more showed partial but significant competition of ^{125}I-MIP-1β binding ($p < 0.05$ compared with binding in the absence of unlabeled ligand, Student's *t* test). *B/T*, bound/total; *B/F*, bound/free.

binding for ^{125}I-MIP-1β that was competitively inhibited by MCP-2 either at room temperature (Fig. 2*A*) or at 4 °C (Fig. 2*B*). Unlabeled MIP-1α also completely inhibited ^{125}I-MIP-1β binding (Fig. 2). These results suggest that MCP-2 has the capacity to interact with CCR5 on HEK293 cells with an efficacy similar to known CCR5 ligands such as MIP-1β and MIP-1α.

Further support for the ability of MCP-2 to interact with CCR5 was obtained with binding studies using ^{125}I-MCP-2. As shown in Fig. 3, ^{125}I-MCP-2 specifically bound to CCR5/293 cells with an estimated K_d in the 5 nM range (Fig. 3*A*). The binding of ^{125}I-MCP-2 to CCR5/293 cells was efficiently inhibited by unlabeled MIP-1β and RANTES (Fig. 3*B*) but less effectively by MCP-1 and MCP-3, which in contrast were able to completely displace MCP-2 binding to CCR1 and CCR2B (14). Thus, MCP-2 possesses binding domains for interaction with CCR5 in addition to CCR1 and CCR2B. This was supported by the observations that although MCP-1, MCP-2 and MCP-3 are all functional ligands for CCR2B, the binding of MCP-1 on CCR2B was poorly competed for by MCP-2 or MCP-3 (14, 23) suggesting differential utilization of certain binding domains on a receptor by multiple ligands. Because promiscuity is a common feature of chemokines and their receptors (24, 25) and receptor activation appears to be dependent on the relative affinity to interact with a ligand, structure analyses and mutagenesis studies are required to more precisely determine the functional epitopes on both ligands and receptors.

We next determined the functional role of MCP-2 on CCR5. Both rhMCP-2 and rhMIP-1β are poor Ca^{2+} mobilizers in monocytes (18, 26), and we were not able to observe significant Ca^{2+} flux in CCR5/293 cells with these two ligands, although a RANTES (120 nM)-induced signal was obtained (data not shown). We therefore utilized chemotaxis assays, which in our previous studies were demonstrated to be very sensitive and reproducible in assessing the activity of a chemokine on a given receptor (14, 19). CCR5/293 cells showed a significant chemotactic response to MCP-2 and MIP-1β (Fig. 4), yielding a typical bell-shaped dose response. MCP-2 was similar in potency to MIP-1β with an almost 6-fold increase over medium control at 1.2–6 nM concentration range, but it was less efficacious (EC_{50}: MCP-2, 0.12 nM; MIP-1β, 0.04 nM, respectively) in inducing CCR5/HEK293 cell migration. Other chemokines known to activate CCR5, such as MIP-1α and RANTES also bound to and induced considerable chemotactic migration of CCR5/293 cells. Although MCP-1 partially displaced MCP-2 binding to CCR5/293 cells, it only bound to and induced migration of CCR2B/293 but not CCR5/293 cells (data not shown). Likewise, MCP-3 also partially displaced MCP-2 binding to CCR5/293 cells; we did not detect significant binding of ^{125}I-MCP-3 to the same cells, nor did it induce migration of CCR5/293 cells at a wide range of concentrations (data not shown). Further proof of the activation of CCR5 by MCP-2 is provided by confocal microscopy (Fig. 5) in which MCP-2 markedly and dose-dependently induced CCR5 internalization in CCR5/293 cells. Another CCR5 ligand RANTES and PMA, a potent protein kinase C activator, similarly caused internalization of CCR5. In contrast, CXC chemokine IL-8, which uses CXCR1 and CXCR2 as functional receptors, did not induce CCR5 internalization (Fig. 5).

The identification of CCR5 as the principal co-receptor for cellular entry by monocyte tropic HIV-1 strains (2–5, 8) and the inhibition of viral fusion and replication by the CCR5 ligands MIP-1α, MIP-1β, and RANTES (9) led us to question whether MCP-2 similarly could inhibit HIV-1 entry and replication. The susceptibility to HIV-1 infection of CCR5/CD4/293 cells was determined in the presence or the absence of MCP-2 (Fig. 6). Preincubation of CCR5/CD4/293 cells with either 800 ng/ml MCP-2 or RANTES completely inhibited HIV-1 entry and subsequent reverse transcription. No reverse transcription products were detected in CCR5/CD4/293 cells treated with HIV-1RF, a T lymphocyte tropic strain (data not shown). CCR5/293 cells without CD4 were not infected by HIV-1ADA. Therefore, the ability of MCP-2 to internalize CCR5 confers on it the

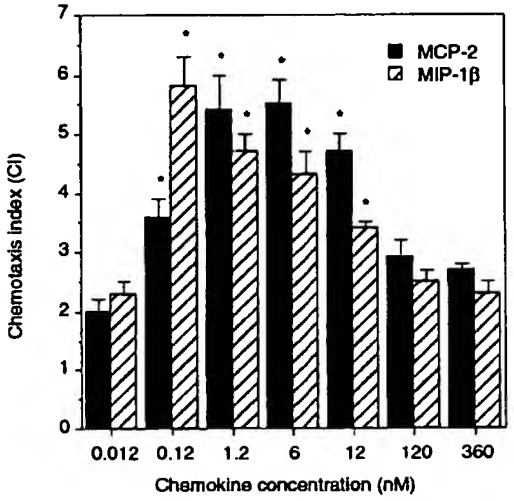

FIG. 4. **Migration of CCR5/293 cells induced by MIP-1β and MCP-2.** The figure presents results (means ± S.D.) from a typical experiment out of five performed. Chemotaxis Index values over 2 are statistically significant ($p < 0.05$) as evaluated by paired Student's *t* test, and * indicates $p < 0.001$.

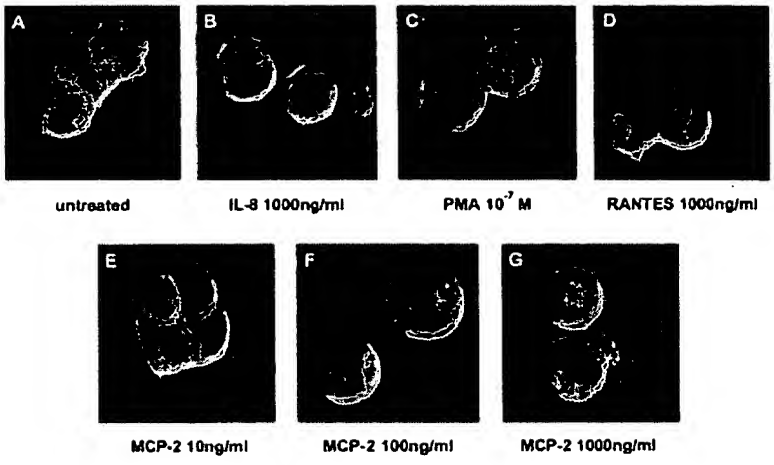

FIG. 5. **Internalization of CCR5 by pretreatment of CCR5/293 cells with MCP-2.** The CCR5/293 cells were treated with different concentrations of MCP-2 and other reagents and then were centrifuged on to slides and permeabilized. The slides were stained with anti-FLAG monoclonal antibody followed by FITC-labeled goat anti-mouse IgG. Nomarski and FITC images were prepared for each specimen using a Zeiss 310 Confocal Laser Scanning Microroscope. Fluorescent images were superimposed on Nomarski images. The images represent untreated cells (*A*) and cells treated with IL-8 (*B*), PMA (*C*), RANTES (*D*), and 10–1000 ng/ml MCP-2 (*E–G*).

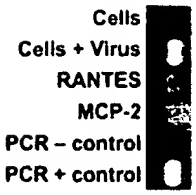

FIG. 6. **Inhibition of HIV-1ADA entry/replication in CCR5/CD4/HEK293 cells by MCP-2.** CCR5/293 cells coexpressing CD4 were preincubated in the presence or the absence of chmeokines for 30 min at 37 °C followed by 24 h of incubation with HIV-1ADA. After washing, cellular genomic DNA was extracted, and HIV-1 reverse transcription products were monitored by PCR with primers corresponding to HIV-1gag region. *Cells*, CCR5/CD4/293 cells without virus; *Cells + Virus*, CCR5/CD4/293 cells incubated with HIV-1ADA; *RANTES*, cells pretreated with 800 ng/ml RANTES then with HIV-1ADA; *MCP-2*, cells pretreated with 800 ng/ml MCP-2 and then with HIV-1ADA; *PCR-control*, CEM-SS cells without HIV-1 infection; *PCR+control*, CEM-SS cells infected with HIV-1_{RF}. Results are from one experiment out of three performed.

ability to interrupt virus entry, with an antiviral activity equivalent to RANTES. MCP-2 also appears to be a natural inhibitor of CD4/CCR5-mediated HIV-1 entry/replication in host cells in addition to MIP-1α, MIP-1β, and RANTES (6, 7, 9).

MCP-2 is constitutively expressed in tumor cells and is inducible by pro-inflammatory cytokines in mononuclear cells and fibroblasts (10, 11). MCP-2 exhibits a broader spectrum of targeted cells, including cells of dendritic phenotype (27). Therefore, MCP-2 may play an important role in recruiting/activating immune cells at inflammatory and neoplastic foci. In the present study, we demonstrated for the first time using cloned CCR5 that MCP-2 is a highly efficacious ligand for this receptor in addition to CCR1 and CCR2B (14). In our preliminary study, HEK293 cells transfected with CCR3, which is a receptor for CC chemokines eotaxin and MCP-4 (28, 29), also were induced to migrate significantly and reproducibly in response to MCP-2 in addition to eotaxin and MCP-4 (data not shown). Thus, MCP-2 appears to use CCR1, CCR2B, CCR5, and possibly CCR3, suggesting it has a more promiscuous functional pattern than other known CC chemokines.

Despite an apparent redundancy in chemokines and their receptor family (24, 25, 30, 31), CC chemokines have been implicated as important mediators of many pathological conditions such as chronic inflammation, immune diseases, neoplasia, and atherosclerosis (30, 31). CCR5 ligands MIP-1α, MIP-1β, and RANTES are also major HIV-1 inhibitors produced by activated mononuclear cells (9). Our current observations extend the functional scope of MCP-2 as a potent inhibitor of monocyte tropic HIV-1 infection in $CD4^+/CCR5^+$ cells. Investigation into the shared and unique functional domains on MCP-2 in comparison with other chemokine ligands will be important in the development of therapeutic approaches to chemokine- and chemokine receptor-mediated pathological states.

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The CC Chemokine I-309 Inhibits CCR8-dependent Infection by Diverse HIV-1 Strains*

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Using a chemokine receptor model based on known receptor sequences, we identified several members of the seven transmembrane domain G-protein superfamily as potential chemokine receptors. The orphan receptor ChemR1, which has recently been shown to be a receptor for the CC chemokine I-309, scored very high in our model. We have confirmed that I-309, but not a number of other chemokines, can induce a transient Ca^{2+} flux in cells expressing CCR8. In addition, the human erythroleukemic cell line K562 responded chemotactically in a dose-responsive manner to this chemokine. Since several chemokine receptors have been shown to be required as coreceptors for HIV-1 infection, we asked whether human immunodeficiency virus type 1 (HIV-1) could efficiently utilize CCR8. Here we show that the CCR8 receptor can serve as a coreceptor for diverse T-cell tropic, dual-tropic, and macrophage-tropic HIV-1 strains and that I-309 was a potent inhibitor of HIV-1 envelope-mediated cell-cell fusion and virus infection. Furthermore, we show by flow cytometry and immunohistochemistry that antibodies generated against the CCR8 receptor amino-terminal peptide cross-reacted with U-87 MG cells stably expressing CCR8, THP-1 cells, HL-60 cells, and human monocytes, a target cell for HIV-1 infectivity *in vivo*.

The chemokines are a diverse group of proteins that play an important role in host defense (1). They are classified into two major groups, CC and CXC, based on the position of the first two of their four invariant cysteines (2). Given their role in host defense, it is no surprise that chemokines and their receptors have been subjected to intense attack by pathogenic organisms. Some viruses have been shown to express viral chemokines and/or chemokine receptors (3–6) presumably as decoy proteins to help subvert the host immune response. Recently, human immunodeficiency virus type-1 (HIV-1)[1] has been shown to utilize chemokine receptors as coreceptors to infect cells. These findings provide new opportunities not only to study HIV-1 pathogenesis but also to develop new anti-viral strategies.

While most HIV-1 strains use CD4 as a primary receptor, a specific chemokine receptor is also required for the membrane fusion reaction subsequent to virus infection. Generally, macrophage-tropic HIV-1 strains utilize CCR5 in conjunction with CD4 (7–11), while the T-cell tropic strains that typically emerge late in the course of the disease utilize CXCR4 (12, 13). In addition, dual-tropic viruses can efficiently utilize both of these receptors (8, 9, 14). Several other chemokine receptors have also been shown to function as coreceptors for a subset of HIV-1 strains, including CCR3 and CCR2b (8, 9). The importance of chemokine receptors for HIV-1 pathogenesis *in vivo* is shown by the finding that approximately 1% of Caucasians are homozygous for a 32-base pair deletion in CCR5 that prevents its transport to the cell surface. These individuals are very highly resistant to virus infection (15–19).

Given the importance of chemokines and their receptors in inflammation, autoimmunity, and the pathogenesis of AIDS, the identification and characterization of these proteins will be important to initiate approaches for therapeutic intervention. We used computer-assisted modelling, based on the sequences of the existing chemokine receptors, to identify potential chemokine receptors from the known data base. Using this approach, we identified a recently described orphan seven transmembrane domain receptor that has been known by several names, including ChemR1, TER-1, GPR-CY6, and CKRL1 (20–22), as a prime candidate for a chemokine receptor. Recently ChemR1 has been shown to be a ligand for the CC chemokine I-309 and is now designated CCR8 (23, 24). We have confirmed that I-309 but not a number of other chemokines can induce a transient Ca^{2+} flux in cells expressing CCR8. In addition, the human erythroleukemic cell line K562-expressed message for CCR8 was able to bind radiolabeled I-309 and responded chemotactically in a dose-responsive manner to this chemokine. We show here that CCR8 is a coreceptor for HIV-1 and that I-309 potently inhibited both HIV-1 envelope-mediated cell-cell fusion and virus infection of cells expressing CD4 and CCR8. Furthermore, we show that antibodies generated against the CCR8 receptor amino-terminal peptide detected CCR8 receptors in U-87 MG cells stably expressing CCR8 in addition to THP-1 and HL-60 cell lines and in human primary monocytes.

MATERIALS AND METHODS

Materials—Unlabeled chemokines were from Peprotech (Rocky Hill, NJ) or from R&D Systems (Minneapolis, MN). ^{125}I-labeled chemokines were obtained from NEN Life Science Products (Boston, MA). Polyclonal antisera to CCR8 was raised in New Zealand White rabbits by

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§§ Supported by a Howard Hughes Medical Institute Predoctoral Fellowship.

[1] The abbreviations used are: HIV, human immunodeficiency virus type-1; RT-PCR, reverse transcriptase polymerase chain reaction; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

subcutaneous and intramuscular injection with the corresponding amino-terminal domain for CCR8 conjugated to KLH. Following primary immunization and six challenges with peptide, CCR8 antiserum from several pooled bleeds was collected and purified over a peptide affinity column. Purified antibody was analyzed by peptide ELISA with a titer of 30,000. Tissue culture media was from Life Technologies, Inc. (Grand Island, NY).

Cloning of CCR8 cDNA—Normal human thymus RNA (CLONTECH) was used as a template for reverse transcription followed by reverse transcriptase polymerase chain reaction (RT-PCR). Based on published sequences, two oligonucleotides, Oligo I (GGAGTGAATGTGTTTTATGTG) and Oligo II (ATTTAGTCTTCATTGATCCTCAC), were synthesized and used to derive an 1135-base pair RT-PCR product corresponding to the open reading frame. The RT-PCR product was cloned into pT7Blue (Novagen) and confirmed by complete DNA sequence analysis. For stable transfectants, the RT-PCR product was cloned into pcDNA3 (Stratagene) expression vector containing G418 selection marker.

Cell Lines and Human Monocytes—Human U87 MG cells expressing CCR8 were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum with 300 μg/ml of G418. Human embryonic kidney (HEK) 293 cells and the Japanese quail fibrosarcoma line QT6-C5 were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated bovine serum. The human erythroleukemic cell line K562, monocytic cell line THP-1, promyelocytic cell line HL-60 clone 15, and the T-cell line Jurkat were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum. Differentiated HL-60 clone 15 cells were generated by treating cells with 0.5 μM butyric acid (Sigma) and 10 ng/ml IL-5 (R&D Systems) as described (23). The murine embryo fibroblast cell line PA317T4 was maintained in Dulbecco's modified Eagle's medium-10 with CD4 expression selected for by the addition of 0.6 mg/ml G418. All cell lines were obtained from the American Type Culture Collection. Human monocytes were purified from peripheral blood of healthy donors. Human buffy coats were obtained from Peninsula Blood Bank (Burlingame, CA), and peripheral blood monocytes were separated using Ficoll-Hypaque as described (25) and two rounds of adherence to plastic in RPMI 1640 medium with 10% fetal bovine serum.

Indirect Antibody Labeling FACS method—Human U-87 MG cells were transfected with the pcDNA3 plasmid that encodes CCR8 as described above. Stable cells were selected with G418. Purified CCR8 polyclonal antisera was incubated with cells expressing the receptors (antibody and cells were diluted 1:750 in PBS without Ca^{2+}/Mg^{2+}; for blocking experiments, antibody and amino-terminal CCR8 peptide were incubated together for 30 min on ice prior to addition to cells). Cells were placed on ice for 30–60 min. Cells were pelleted with the supernatant removed, placed back in PBS, and washed twice. Cells were then incubated with goat anti-rabbit-FITC-conjugated antibody (diluted 1:1000 in PBS) on ice in the dark for 30 min. Cells were pelleted and washed as above. Controls for indirect labeling were (*a*) no primary, secondary-FITC only, (*b*) pre-immune serum with secondary, (*c*) nontransfected cell staining, (*d*) specific blocking with amino-terminal peptide, and (*e*) cross-reactivity with CXCR1, CXCR2, CCR1, and CCR5 stably expressing cell lines.

Immunohistochemistry—Cultured cells were grown on 8-well, plastic (Thermanox™) chamber slides and stained as described previously (26), with the exception of adding Triton X-100 to buffers to maintain membrane integrity. Cells were viewed on a Zeiss Axioskop and photographed with an attached Fuji HC-2000, three-chip CCD digital camera. Images were first edited in Adobe Photoshop 3.0 to enhance contrast and white balance and then printed on a Fuji Pictrography 3000 digital printer.

Sequence Comparison Using Gibbs Sampling—We used the MACAW (Multiple Alignment Construction and Analysis Workbench) computer program running on a Macintosh platform to generate a chemokine receptor model based on the nine primary amino acid sequences for CCR1–5 and CXCR1–4 to detect blocks of similarity. Two blocks corresponding to positions 70–80 (SIYLLNLAISDLLFLFTLPFW) and 286–308 (TEVIAYTHCCVNPVIYAFVGERF) of CCR1 were found, and similar blocks were located at comparable sites in each of the chemokine receptors. This model was then used to search the TREMBL data base to find potential chemokine receptors.

Transfection—QT6 and HEK293 cells were transiently transfected with plasmids encoding the CCR8 receptor by the calcium phosphate precipitation method (8). After overnight expression, cells were removed from the plate with 1 mM EDTA, centrifuged, and resuspended in PBS for binding and biologic studies.

Chemokine Binding Studies—For binding assays cells (5×10^5 cells/ml) were incubated in PBS (0.2 nM) and varying concentrations of unlabeled ligands at room temperature for 30 min. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously (27). Nonspecific binding was determined in the presence of 1 μM unlabeled ligand.

CXCR1	DTFLLHLAVADTLLVLTLPLW	YAGALLLACISFDRYLNIVHAT
CXCR2	DVYLLNLALADLLFALTLPIW	YSGILLLACISVDRYLAIVHAT
CXCR3	DVYLLNLALADLLFALTLPIW	YSGILLLACISVDRYLAIVHAT
CXCR4	DKYRLHLSVADLLFVITLPFW	YSSVLILAFISLDRYLAIVHAT
CCR1	SIYLLNLAISDLLFLFTLPFW	YSEIFFIILLTIDRYLAIVHAV
CCR2	DIYLLNLAISDLLFLITLPLW	FGGIFFIILLTIDRYLAIVHAV
CCR3	NIYLLNLAISDLLFLVTLPFW	YSEIFFIILLTIDRYLAIVHAV
CCR4	DVYLLNLAISDLLFVFSLPFW	YSGIFFVMLMSIDRYLAIVHAV
CCR5	DIYLLNLAISDLFFLLTVPFW	FGGIFFIILLTIDRYLAVVHAV
CCR6	DVYLVNMAIADILFVLTLPFW	NCGMLLLTCISMDRYIAIVQAT
CCR7	DTYLLNLAVADILFLLTLPFW	FSGMLLLLCISIDRYVAIVQAV
CCR8	DVYLLNLALSDLLFVFSFPFQ	YSSMFFITLMSVDRYLAVVHAV

FIG. 1. **Identification of a profile of chemokine receptors using the program MACAW.** The sequences shown as block 1 and block 2 correspond to regions of MACAW-detected sequence similarity among CXCR1-CXCR4 and CCR1-CCR7. The sequences of the blocks were used as query sequences for the BLAST algorithm against the TREMBL data base. A repeatedly high scoring sequence was CCR8, which is shown aligned *below* CCR7.

Ca^{2+} Flux; assays—CCR8 was expressed in HEK293 cells by transient transfection. Cells were loaded with 2.5 μM Fura-2/AM (Molecular Probes) at 37 °C in the dark for 1 h, allowed to efflux for 15 min in PBS, resuspended in PBS with Ca^{2+}/Mg^{2+}, and warmed at 37 °C for 10 min before measurement of ligand response. Ca^{2+} mobilization was measured in an Aminco-Bowman Luminescence Spectrometer in a constantly stirring cuvette in a volume of 1.5 ml. Thrombin activating peptide-4 was kindly provided by Lawrence Brass (University of Pennsylvania) and was used at a final concentration of 27 μM.

Chemotaxis—K562 cell migration was examined using a 48-well microchemotaxis assay as described previously (28). The results were expressed as the number of migrating cells per three high power fields (± S.E.).

Gene Reporter Fusion Assays—Cell-cell fusion was monitored by a luciferase-based gene reporter assay (10, 29). PA317T4 cells that stably express human CD4 were transfected with luciferase-T7 and the desired coreceptor. T7 RNA polymerase and envelope proteins were introduced into effector HeLa cells by recombinant vaccinia viruses (29). To initiate fusion, target and effector cells were mixed in 24-well plates at 37 °C. To assess the ability of I-309 to inhibit cell-cell fusion, target cells were incubated with I-309 for 30 min at 37 °C prior to mixing with effector cells. After 8 h, cells were lysed and assayed for luciferase activity.

Infection Studies—Viral stocks were prepared by transfecting HEK293 cells with plasmids encoding the desired envelope and the NL4–3 luciferase virus backbone (pNL-Luc-E-R-) (10). For infection, U87-MG cells were plated in 24-well plates and transfected with pT4 and the desired coreceptor. The medium was changed after 6 h, and cells were allowed to express overnight. Cells were infected the next day with 500 μl of viral supernatant. Media was changed the following day, and 0.5 ml of additional media was added 1 day prior to harvest of cells. Cells were lysed at 4 days post-infection by resuspension in 150 μl of 0.5% Nonidet P-40/PBS, and 50 μl of the resulting lysate was assayed for luciferase activity.

RESULTS AND DISCUSSION

Chemokine receptors belong to a superfamily of seven transmembrane domain proteins that signal through coupled heterotrimeric G proteins (30). At the latest count, well over 600 members of this superfamily have been identified and classified into families (31). However, a number of cloned serpentine receptors exist for which no endogenous ligands have been identified. To determine whether we could assign any of the orphan receptors in the TREMBL data base to the chemokine receptor family, we looked for regions of local similarity (blocks) based on the protein sequences of the receptors for CC and CXC chemokines, CCR1 through CCR5 and CXCR1 through CXCR4, using a Gibbs sampling strategy (MACAW) (32). We located two significant blocks corresponding to the second and third transmembrane segments (Fig. 1). We then used the program BLAST (33) to identify sequences matching the 21

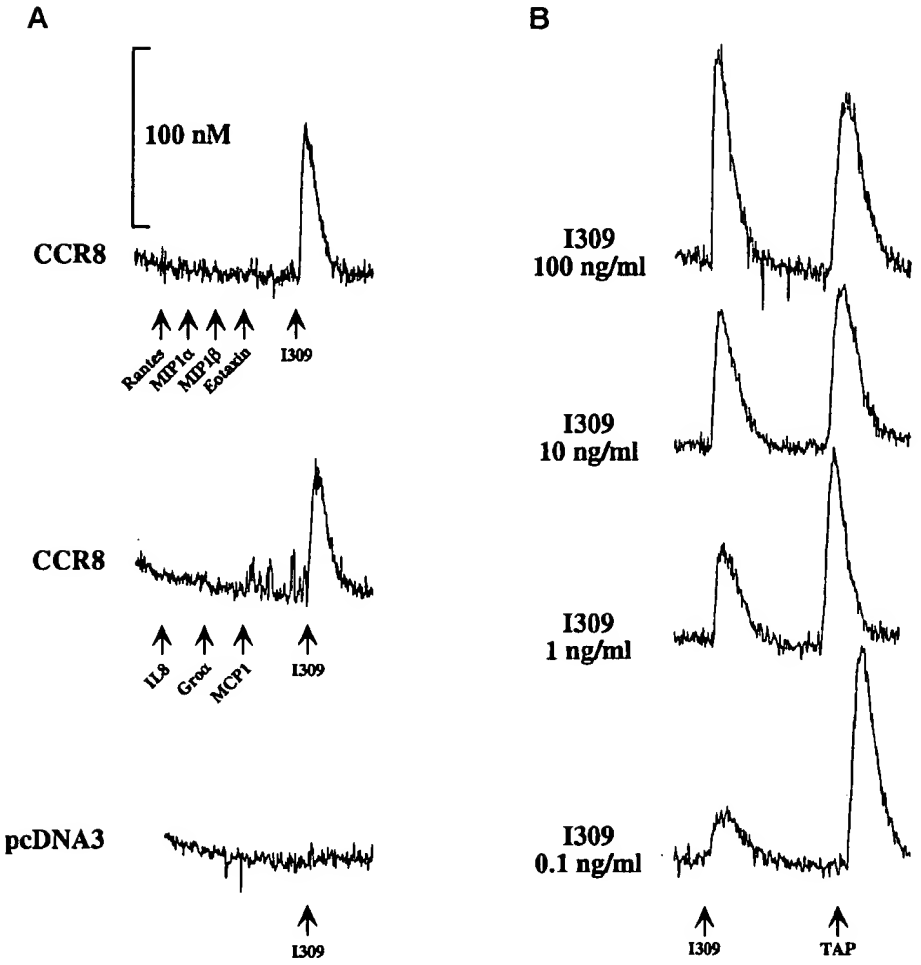

FIG. 2. **I-309 mobilizes intracellular Ca^{2+} in cells transiently expressing the CCR8 receptor.** *A*, HEK293 cells transfected with pcDNA3-CCR8 or the control plasmid pcDNA3 were loaded with Fura-2 and stimulated with 100 nM of the indicated chemokines. *B*, HEK293 cells transfected with pcDNA3-CCR8 were loaded with Fura-2 and stimulated with increasing concentrations of I-309. Dilutions below 0.1 ng/ml I-309 did not yield signals above background. Activation of the thrombin receptor by thrombin-activating peptide-4 (TAP), in both *panel A* (data not shown) and *panel B*, indicated that cells were fully capable of signaling.

amino acid block near the second transmembrane segment and the 22 amino acid block near the third transmembrane segment. In addition to the known chemokine receptors, the highest scoring sequences represented orphan receptors. The orphan receptor that scored the highest was a protein known as ChemR1 where 23/23 amino acids (285–307) matched the block (Fig. 1). Although BLAST queries using the entire amino acid sequence of a particular chemokine receptor sequence (*e.g.* CCR1) also identified orphan receptors, we noted that known chemokine receptors (*e.g.* CCR5) scored worse than members of other families (*e.g.* angiotensin receptors) using this approach, and therefore, we considered its predictive value to be low.

ChemR1 was the highest scoring orphan receptor in our analysis. It was recently identified as a receptor for the CC chemokine I-309 (23, 24) and is now designated CCR8. To confirm the ligand specificity of CCR8, we tested the ability of a number of CXC and CC chemokines including RANTES, MIP-1α, MIP-1β, Eotaxin, IL-8, MGSA, IP10, MCP-1, MCP-3, and I-309 to elicit an increase in Ca^{2+} flux in HEK293 cells transiently expressing the CCR8 receptor. As expected, only I-309 gave a transient calcium response (Fig. 2*A*). The EC_{50} for calcium mobilization by I-309 was approximately 1 ng/ml (Fig. 2*B*).

I-309 is a CC chemokine that was originally identified by subtractive hybridization of a cDNA library from an IL-2-dependent T-cell line (25). Recently, I-309 has been shown to protect murine T cell lymphomas against dexamethasone-induced apoptosis (34). Beyond these few reports, little is known regarding the real physiological role of I-309. It was originally described as a factor secreted by activated T cells that was able to stimulate the migration of human monocytes (35). Therefore, we tested the ability of I-309 to induce chemotaxis in a number of cell lines. We found that K562, which we and others have shown expresses CCR8 (20), and several human neuroblastoma cell lines[2] stimulated with I-309, demonstrated a significant dose-dependent chemotactic response *in vitro* (Fig. 3). These cell lines exhibited typical bell-shaped dose-response curves in their migration, and the optimal I-309 concentration was between 10–100 ng/ml. Based on the finding that K562 cells expressed transcripts for CCR8 and responded chemotactically to I-309, we tested the ability of these cells to bind radiolabeled I-309. Although K562 cells incubated with ^{125}I-labeled I-309 specifically bound the radiolabeled chemokine, the total binding was low (4200 cpm/10^6 cells), and the nonspecific binding ranged from 50 to 60% of the total.

Recently, members of the chemokine receptor family have been shown to serve as coreceptors for HIV-1. Macrophage-

[2] D. Taub, unpublished data.

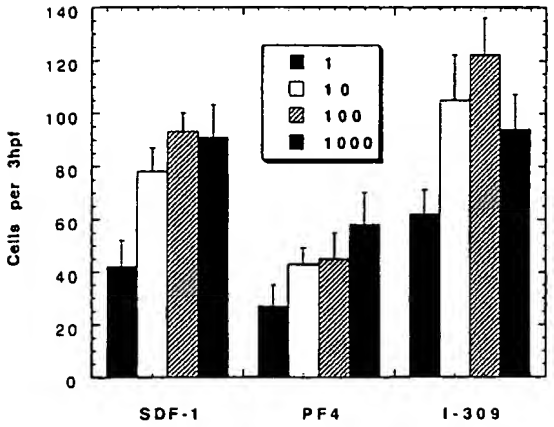

FIG. 3. **I-309 induces cell migration in K562 cells.** Cells were tested for their ability to migrate in response to various concentrations of the CXC chemokines IL-8 and PF4 and the CC chemokine I-309, as described under "Materials and Methods." The results are expressed as the number of cells per three high power fields (*3hpf*) ± S.E.

tropic strains of HIV-1 use mainly CCR5 (7–11), T-cell line tropic strains of HIV-1 use CXCR4 (12), and some viruses can use other receptors including CCR2b, CCR3, STRL33, and V28 (8, 9, 14, 36, 37). Thus, to target chemokine receptors therapeutically with small molecule antagonists, it will be important to define the range of chemokine receptors that can be utilized by HIV-1 as coreceptors for invasion. We wanted to determine whether CCR8 could serve as a coreceptor for HIV-1 and whether I-309 could inhibit this. Thus, we used a cell-cell fusion assay in which HeLa cells expressing the desired HIV-1 envelope protein and T7 polymerase are mixed with target cells expressing CD4, a coreceptor, and luciferase under control of the T7 promoter(8). If cell-cell fusion occurs, luciferase is produced as a consequence of cytoplasmic mixing. To determine if I-309 could inhibit HIV-1 envelope-mediated cell-cell fusion, cells expressing the ADA (macropohage-tropic) or BK132 (T-cell tropic) envelope proteins were mixed with cells expressing CD4 and CCR8. As shown in Fig. 4*A*, the ADA and BK132 envelope proteins mediated fusion with cells expressing CD4 and CCR8. Fusion was strongly inhibited by I-309 in a dose-dependent manner, providing further evidence that I-309 is a CCR8 ligand (Fig. 4*A*). I-309 did not block fusion when either CCR5 or CXCR4 were used as coreceptors (data not shown).

To determine if I-309 could also inhibit virus infection, cells expressing CD4 and CCR8 were incubated with I-309 prior to infection with HIV-1 ADA, which uses both CCR5 and CCR8 as coreceptors. As shown in Fig. 4*B*, I-309 strongly inhibited infection by HIV-1 when cells expressed CCR8 but not when CCR5 was expressed. Thus, I-309 inhibits CCR8-dependent envelope-mediated cell-cell fusion and virus infection.

Since we have shown that CCR8 is a coreceptor for HIV-1, it will be important to establish the relevance of this receptor in the pathogenesis of AIDS by (*a*) identifying cells that express the receptor, (*b*) ascertaining if they are targets of the virus, and (*c*) determining the regulation of the receptor in these cell types. In addition, it will be interesting to examine cells and tissues of AIDS patients to determine whether CCR8 plays a real role in HIV-1 transmission. To answer these and other important questions will require immunological approaches using CCR8 receptor antibodies. Since antibodies to a number of chemokine receptors have been successfully raised using amino-terminal peptides as immunogens (26), we generated antibodies to CCR8 using a similar approach. The purified anti-CCR8 polyvalent antisera were used to stain transfectants expressing CCR8 receptors. The indirect immunofluorescence

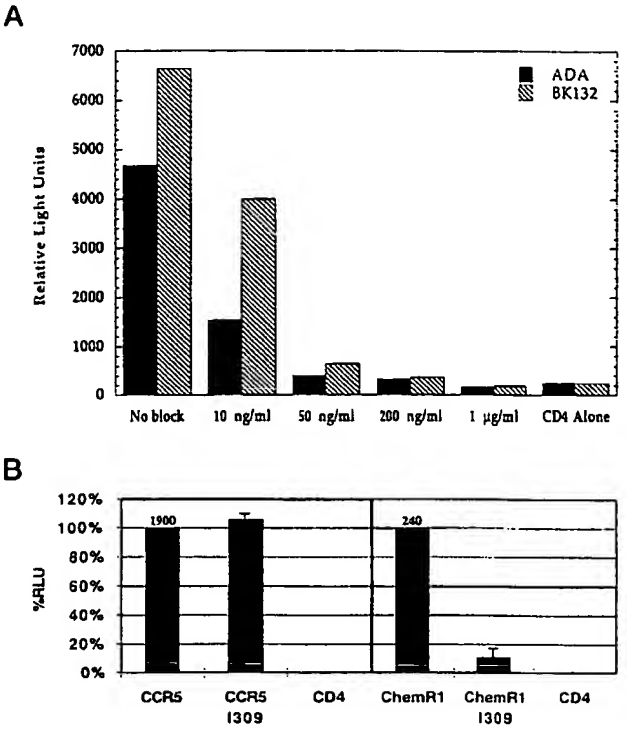

FIG. 4. **CCR8-dependent cell-cell fusion and virus infection are inhibited by I-309.** *A*, cell-cell fusion was determined by mixing murine PA317T4 cells (which express human CD4) expressing CCR8 and containing the luciferase gene under the control of the T7 promoter with HeLa cells expressing T7 polymerase and either ADA (macropohage-tropic) or BK132 (T-cell tropic) envelope protein. I-309, at the indicated concentrations, was incubated with target cells for 30 min at 37 °C prior to mixing with effector cells. The degree of cell-cell fusion was determined by measuring relative light units 8 h after mixing. I-309 showed no inhibitory effect against CCR5- or CXCR4-dependent cell-cell fusion at 1 µg/ml (data not shown). *B*, U87-MG CCR8 cells were plated in 24-wells and transfected with pT4 (encoding CD4) and the desired coreceptor. I-309 was added at a concentration of 100 ng/ml 30 min prior to infection with 500 µl of viral supernatant containing a luciferase-reporter virus bearing the ADA envelope protein. Cells were lysed at 4 days post-infection, and the lysate was assayed for luciferase activity. *RLU*, relative light units.

was analyzed by flow cytometry. As shown in Fig. 5, the polyvalent CCR8 receptor antibodies bound to the native receptor expressed by these transfectants. This binding was specific since it was inhibited by the addition of the CCR8 amino-terminal peptide, and pre-immune serum did not stain these cells (Fig. 5*A*). The antibodies were specific for CCR8 since the antibodies did not recognize CXCR1-, CXCR2-, CCR1-, or CCR5-transfected cells or untransfected U87-MG parental cells (data not shown). To further investigate the ability of our CCR8 antisera to recognize other cell types that may express the receptor, we carried out FACS analysis of three human cell lines, THP-1, HL-60, and Jurkat, and also examined human monocytes. The human monocytic cell line THP-1 has been reported to respond chemotactically to the murine homolog of I-309, TCA3, (38) while I-309 has been shown to induce an increase in intracellular Ca^{2+} mobilization in human HL-60 cells induced with IL-5 and butyric acid (23). Human Jurkat cells were recently shown to be negative for CCR8 mRNA (21). As expected, FACS analysis of these cell lines revealed CCR8 expression on THP-1 and HL-60 cells while Jurkat cells showed no staining (Fig. 5, *B-E*). The staining was specific since it was blocked by the CCR8 amino-terminal peptide. Interestingly, we find that unstimulated HL-60 cells cross-react with the CCR8 antibodies (Fig. 5*D*), consistent with this they also bind ^{125}I-

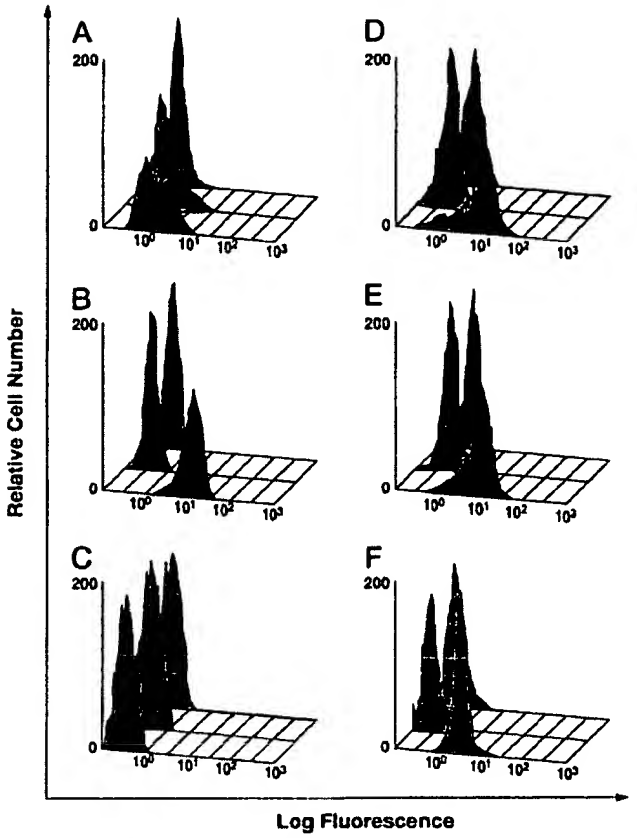

FIG. 5. **Antibodies to CCR8 amino-terminal peptides recognize the CCR8 receptor by flow cytometric analysis.** Several cell lines were incubated with CCR8 receptor antibodies in the presence and absence of CCR8 amino-terminal peptide and examined by flow cytometry. U87 MG cells stably expressing the CCR8 receptor (*A*), THP-1 (*B*), Jurkat (*C*), HL-60 clone 15 cells (*D*), HL-60 clone 15 cells treated with IL-5 and butyric acid (*E*), and primary human monocytes (*F*). *Red* shifts represent incubation of cells with anti-CCR8 antibodies and goat anti-rabbit-IgG FITC secondary antibody. *Green* shifts represent incubation of cells with pre-immune sera and goat anti-rabbit-IgG FITC secondary antibody. *Purple* shifts represent incubation of cells with anti-CCR8 antibodies in the presence of CCR8 amino-terminal peptide followed by incubation with goat anti-rabbit-IgG FITC secondary antibody.

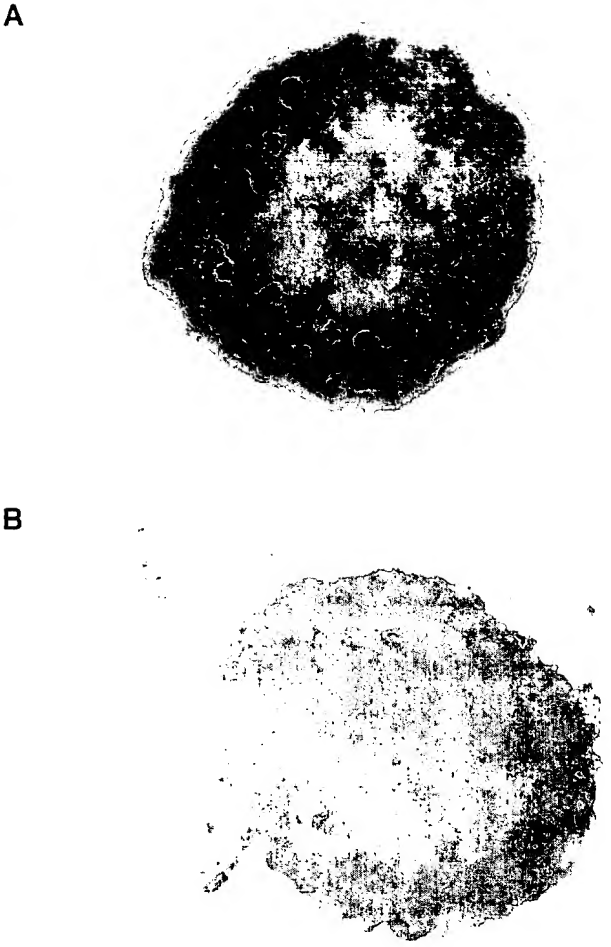

FIG. 6. **Antibodies to CCR8 amino-terminal peptides immunohistochemical stain cells expressing the CCR8 receptor.** Human U-87 MG glioblastoma cells transfected with CCR8 receptors were stained with antibodies to CCR8 in the absence (*A*) and presence (*B*) of peptides specific for the antibody.

labeled I-309.[3] Tiffany *et al.* (23) have shown that only the differentiated HL-60 cells respond to I-309, which may be due to up-regulation and expression of intracellular proteins that allow CCR8 to transduce signals upon binding of ligand. Since human monocytes have clearly been shown to respond to I-309 by chemotaxis and by induction of cell migration (35), we examined these cells with the CCR8 antibodies. As seen in Fig. 5*F*, monocytes stained strongly with the antibodies.

To further test for chemokine receptor expression, human U-87 MG cells stably expressing CCR8 were immunohistochemically stained using antibodies to the CCR8 receptor. The cells were plated in 8-well chamber slides and stained as described previously (26). As can be seen in Fig. 6*A*, the U-87 MG cells stained strongly with the CCR8 antisera. However, not all of the cells stained with the antisera, which is consistent with the partial shift observed by indirect FACS analysis (Fig. 5*A*). The specificity of the CCR8 receptor staining was established by demonstrating that peptides specific for the antibodies could appropriately block antibody staining (Fig. 6*B*) and that specific staining was not observed with irrelevant monoclonal and polyclonal antibodies (data not shown).

[3] J. Hesselgesser, unpublished data.

Our findings that CCR8 functions as a coreceptor for diverse HIV-1 strains makes this molecule potentially relevant for viral pathogenesis. The ability of I-309 to inhibit CCR8-dependent virus infection should make it possible to determine if HIV-1 strains utilize this chemokine receptor for infection of relevant target cells *in vivo*. Indeed, we have clearly shown that CCR8 is expressed in human monocytes as a major target cell of HIV-1. The availability of antibodies to immunologically detect and characterize CCR8 should also make it possible to determine the relevance of this coreceptor in HIV-1 transmission.

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